

Mohammadreza Baghaban Eslaminejad ·
Samaneh Hosseini *Editors*

Cartilage: From Biology to Biofabrication



Springer

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Mohamadreza Baghaban Eslaminejad
Cell Science Research Center
Royan Institute
Tehran, Iran

Samaneh Hosseini
Cell Science Research Center
Royan Institute
Tehran, Iran

ISBN 978-981-99-2451-6

ISBN 978-981-99-2452-3 (eBook)

<https://doi.org/10.1007/978-981-99-2452-3>

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The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

Cartilage is the main component of synovial joints, which is characterized by a highly organized extracellular matrix (ECM). This tissue has limited self-repair capacity and its minor lesions may result in progressive damage on joint such as osteoarthritis (OA). Cartilage defects affect patients of all age groups. Despite being an apparently uncomplicated tissue, cartilage repair has been an unattainable goal due to avascularity of this tissue.

This book provides up-to-date information regarding the structure, function, cellular and molecular biology of the cartilage tissue. In 18 chapters, the book covers the traditional therapies and also novel strategies that accelerates cartilage formation. Recent advances in biomaterials sciences, regenerative medicine, and fabrication technology such as bioprinting with a discussion of their successes, limitations, and hopes for future for cartilage regeneration are taken into consideration. The numerous translational and clinical studies in cartilage tissue engineering have also been discussed to address current advancements and challenges of fabricated constructs as well as stem cells used for clinical applications. It is hoped that this book will be useful for scientists, researchers, engineers, and professionals who are interested and involved in cartilage tissue development and regeneration.

We are deeply indebted to all the authors for their energetic contribution and sincerely appreciate their participation and help with this undertaking. A special word of thank goes to Niloofar Kalantari and Samaneh Adhami for their assistance with data collection. We are extremely thankful to the Springer team, specifically Dr. Naren Aggarwal, Dr. Bhavik Sawhney, and Dr. Nandhini Viswanathan for their administrative support, help, and coordinating the production and publication of this book.

Tehran, Iran

Mohamadreza Baghaban Eslaminejad
Samaneh Hosseini

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Editors and Contributors

About the Editors

Mohamadreza Baghaban Eslaminejad is the Head of Bone and Cartilage group at Royan Institute for Stem Cell Biology and Technology, Tehran, Iran. He founded the adult stem cell lab in 2004 and started his research on mesenchymal stem cells as a potent tool in cell therapy for orthopedic diseases. He has supervised numerous investigations in basic sciences, preclinical and some clinical trials related to regenerative medicine. He has been appreciated with over six major national and international awards notably, Royan International Research Award for Reproductive Biomedicine and Stem Cell Researches (2006), the award for the Leading Researcher of Tehran (2007), the Best Researcher Award from Razi Medicine Festival (2009), the award from National Festival for Commemorating Distinguished Researchers (2010), the award for the exemplary scientist and staff of the year 2015 by ACECR (The Academic Center for Education, Culture and Research, Iran). In 2018 and 2019, he was included in the list of scientific leaders of Iran. Springer Nature selected one of the Eslaminejad chapter books with the title of “3D Printing in Dentistry” as one of 2020 highlighted research. He has published more than 250 articles and 180 abstracts in peer-reviewed journals and has participated in national and international conferences. He has also contributed to 20 chapters and authored three books.

Samaneh Hosseini is an Assistant Professor at Cell Engineering Department at Royan Institute, Tehran, Iran. Her research interest is in Tissue Engineering with specialization on biomineralization, mimetic peptides, cell-based tissue engineering for regeneration of bone and cartilage tissues. She has conducted several research projects in both basic sciences and preclinical studies in the fields of tissue engineering and regenerative medicine. She has served as a referee for a number of international journals including, Stem Cell Research and Therapy (UK), International Journal of Biological Macromolecules (Netherlands), Journal of Cellular physiology (USA), Cell Proliferation (UK), and Cell Journal (Iran). She has more than 10 years of teaching experience in tissue engineering and nanobiotechnology. She has also published more than 30 research articles in the peer-reviewed international journal and served as an author or co-author in several book chapters. One of her

publications, *3D Printing in Dentistry* was one of the Springer Nature 2020 highlights.

Contributors

Amirreza Ahmadiasl Department of Cell Engineering, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Department of Biomedical Engineering, Faculty of Chemical Engineering, Tarbiat Modares University, Tehran, Iran

Muhammad Rajaei Ahmad Mohd Zain Department of Orthopaedics, School of Medical Sciences, Health Campus, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia

Babak Akbari Life Science Engineering Department, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran

Viktorija Aleksiuk Department of Regenerative Medicine, State Research Institute Centre for Innovative Medicine, Vilnius, Lithuania

Amirabbas Amini Department of Materials Engineering, Science and Research Branch, Islamic Azad University, Tehran, Iran

Hiroshi Asahara Department of Molecular Medicine, Scripps Research, La Jolla, CA, USA

Department of Systems Biomedicine, Tokyo Medical and Dental University, Tokyo, Japan

Maryam Azlan School of Health Sciences, Health Campus, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia

Payam Baei Department of Cell Engineering, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Fatemeh Bagheri Department of Biotechnology, Faculty of Chemical Engineering, Tarbiat Modares University, Tehran, Iran

Valentina Basoli AO Research Institute Davos, Davos, Switzerland

Eiva Bernotiene Department of Regenerative Medicine, State Research Institute Centre for Innovative Medicine, Vilnius, Lithuania

Yanli Cai NUS Centre for Additive Manufacturing (AM.NUS), National University of Singapore, Singapore, Singapore

Mohamadreza Baghaban Eslaminejad Department of Cell Engineering, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Shahab Faghihi Stem Cell and Regenerative Medicine Group, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

Mahsa Fallah Tafti Stem Cell and Regenerative Medicine Group, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

Nesa Fani Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

SinaCell Research and Production Co., Pardis, Iran

Sara Farahi Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Yuta Fujii Department of Systems Biomedicine, Tokyo Medical and Dental University, Tokyo, Japan

Soo Wah Gan NUS Centre for Additive Manufacturing (AM.NUS), National University of Singapore, Singapore, Singapore

Mahsa Ghasemzad Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Department of Molecular Cell Biology-Genetics, Faculty of Basic Sciences and Advanced Technologies in biology, University of Science and Culture, Tehran, Iran

Mahsa Ghorbaninejad Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Reihaneh Golru Faculty of Biological Sciences, Alzahra University, Tehran, Iran

Sibylle Grad AO Research Institute Davos, Davos, Switzerland

Derrick Guo Department of Orthopaedic Surgery, Woodlands Health Campus, Singapore, Singapore

Department of Orthopaedic Surgery, Sengkang General Hospital, Singapore, Singapore

Samaneh Hosseini Department of Cell Engineering, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Farzaneh Jabbari Nanotechnology and Advanced Materials Department, Materials and Energy Research Center (MERC), Tehran, Iran

Shahrbano Jahangir AO Research Institute Davos, Davos, Switzerland

Ursule Kalvaityte Department of Regenerative Medicine, State Research Institute Centre for Innovative Medicine, Vilnius, Lithuania

Irfan Khan Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan

Fatemeh Khodabandehloo Department of Genetics and Advanced Medical Technology, Faculty of Medicine, AJA University of Medical Sciences, Tehran, Iran

Eng Hin Lee Tissue Engineering Program, Life Sciences Institute, National University of Singapore, Singapore, Singapore
Department of Orthopaedic Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

Kaihu Li AO Research Institute Davos, Davos, Switzerland
Department of Orthopaedics, Xiangya Hospital of Central South University, Changsha, China
Department of Orthopaedics, The Second Xiangya Hospital of Central South University, Changsha, China

Zhen Li AO Research Institute Davos, Davos, Switzerland

Heng An Lin Department of Orthopaedic Surgery, Sengkang General Hospital, Singapore, Singapore

Daryl Jimian Lin Tissue Engineering Program, Life Sciences Institute, National University of Singapore, Singapore, Singapore
Department of Orthopaedic Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

Wen Feng Lu NUS Centre for Additive Manufacturing (AM.NUS), National University of Singapore, Singapore, Singapore
Department of Mechanical Engineering, National University of Singapore, Singapore, Singapore

Sharareh Mahdavi Research Operations, The Hospital for Sick Children, Toronto, ON, Canada

Sara Malih Department of Radiology, University of Wisconsin-Madison, Madison, WI, USA
Department of Medical Biotechnology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

Shohreh Mashayekhan Department of Chemical and Petroleum Engineering, Sharif University of Technology, Tehran, Iran

Farzaneh Mirzaeian Stem Cell and Regenerative Medicine Group, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

Ali Mobasher Department of Regenerative Medicine, State Research Institute Centre for Innovative Medicine, Vilnius, Lithuania

Research Unit of Health Sciences and Technology, Faculty of Medicine, University of Oulu, Oulu, Finland

World Health Organization Collaborating Center for Public Health Aspects of Musculoskeletal Health and Aging, Université de Liège, Liège, Belgium

Department of Joint Surgery, First Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong Province, China

Nur Azira Mohd Noor School of Health Sciences, Health Campus, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia

Reza Naeimi Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

Ryo Nakamichi Department of Orthopaedic Surgery, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan

Department of Molecular Medicine, Scripps Research, La Jolla, CA, USA

Department of Systems Biomedicine, Tokyo Medical and Dental University, Tokyo, Japan

Nahid Nasiri Department of Photo Healing and Regeneration, Medical Laser Research Center, Yara Institute, Academic Center for Education, Culture and Research (ACECR), Tehran, Iran

Asma Abdullah Nurul School of Health Sciences, Health Campus, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia

Zachariah Gene Wing Ow Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

Jolita Pachaleva Department of Regenerative Medicine, State Research Institute Centre for Innovative Medicine, Vilnius, Lithuania

Deepak Raghothaman Department of Orthopaedic Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

Harishini Rajaratnam School of Health Sciences, Health Campus, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia

Faiza Ramzan Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan

Asmat Salim Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan

Parisa Samadi Department of Operating Room, School of Nursing and Midwifery, ShahidBeheshti Hospital, Isfahan University of Medical Sciences, Isfahan, Iran

Marzieh Savari Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

Mina Shahnazari Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

Mohsen Sheykhasan Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

Department of Mesenchymal Stem Cells, The Academic Center for Education, Culture and Research, Qom, Iran

Niloofer Shokrollah Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

Nazmul Huda Syed School of Health Sciences, Health Campus, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia

Leila Taghiyar Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Maryam Talebi Jouybari Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Department of Developmental Biology, University of Science and Culture, Tehran, Iran

Saba Taheri Department of Embryology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

Lobat Tayebi Marquette University School of Dentistry, Milwaukee, WI, USA

Iлона Uzieliene Department of Regenerative Medicine, State Research Institute Centre for Innovative Medicine, Vilnius, Lithuania

Raminta Vaiciuleviciute Department of Regenerative Medicine, State Research Institute Centre for Innovative Medicine, Vilnius, Lithuania

Xinwei Wang Department of Rehabilitation Medicine, Nanjing First Hospital, Nanjing Medical University, Nanjing, Jiangsu, China

Merng Koon Wong Department of Orthopaedic Surgery, Sengkang General Hospital, Singapore, Singapore

Keng Lin Wong Department of Orthopaedic Surgery, Sengkang General Hospital, Singapore, Singapore

Musculoskeletal Sciences Academic Clinical Programme, Duke–NUS Graduate Medical School, Singapore, Singapore

Peng Xia Department of Rehabilitation Medicine, Nanjing First Hospital, Nanjing Medical University, Nanjing, Jiangsu, China

Zheng Yang Tissue Engineering Program, Life Sciences Institute, National University of Singapore, Singapore, Singapore
Department of Orthopaedic Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

Ching-Chiuann Yen NUS Centre for Additive Manufacturing (AM.NUS), National University of Singapore, Singapore, Singapore
Division of Industrial Design, National University of Singapore, Singapore, Singapore

Parisa Zadehnajar Life Science Engineering Department, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran



Introduction to Cartilage Tissue: Development, Structure, and Functions

1

Nahid Nasiri, Saba Taheri, Samaneh Hosseini,
and Mohamadreza Baghaban Eslaminejad

Abstract

Articular cartilage (AC) is an anisotropic and poro-viscoelastic form of connective tissue, which has a key role in skeletal movement in mammals by frictionless and wear-resistant bearing behaviors. The highly organized structure of AC in the form of different micro- and macrostructures and zonation indicates its unique properties and tissue-specific function required to enable lifetime movement. AC development, growth, and maturation occur to achieve specific size, structure, and function and also to serve as a template for bone development and growth. Mature AC has great compressive, shear, and tensile properties as well as swelling behavior by which it can bear mechanical forces, minimize friction, and reduce transmitted forces on the underlying bone. Various experimental approaches have been employed to describe the dynamic changes during AC growth and maturation, both on biologic and on biomechanical characteristics. Such studies can explain relevant mechanisms which can be used in regenerative medicine and improvement of tissue engineering methods and restore tissue

N. Nasiri

Department of Photo Healing and Regeneration, Medical Laser Research Center, Yara Institute, Academic Center for Education, Culture and Research, ACECR, Tehran, Iran

S. Taheri

Department of Embryology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

S. Hosseini (✉) · M. Baghaban Eslaminejad (✉)

Department of Cell Engineering, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

e-mail: hosseini.samaneh@royaninstitute.org; eslami@royaninstitute.org

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M. Baghaban Eslaminejad, S. Hosseini (eds.), *Cartilage: From Biology to Biofabrication*, https://doi.org/10.1007/978-981-99-2452-3_1

degeneration. This study summarizes AC composition, structure, and function through different stages of AC development and growth which are of great importance to the tissue regeneration and tissue engineering approaches.

Keywords

Articular cartilage · Extracellular matrix · Chondrocyte · Tissue development · Tissue maturation

1.1 Introduction

Human articular (hyaline) cartilage (AC) is a unique and specialized biphasic tissue that in a healthful state permits almost frictionless movement across its surface. In any joint, the interface between bones is provided by AC which minimizes the friction and transfers the load into the underlying bone. This nonhomogeneous, poro-viscoelastic connective tissue can serve as a load-bearing cushion in synovial joints and is therefore fundamental for mammalian skeletal development. The design of AC and its mechanical properties permit a lifetime of significant shear force and compressive loads (Brody 2015; Li et al. 2021).

Water and extracellular matrix (ECM) form the main AC component which present high elasticity and strength, required for prolonged and repetitive movement. AC also contains chondrocytes as the only cartilage cell population. After formation, during skeletal maturity period, AC maturation occurs through a tissue remodeling process resulting in a heterogeneous composition with four well-defined and differentiated layers (Eyre 2004). Both the ECM component and chondrocytes have specific concentration and organization in each distinct mature AC zonation.

However, despite its important role, AC has restricted capacity for regeneration mainly due to low cell density, low proliferative activity of chondrocytes, high tendency of the chondrocytes for de-differentiation, and also the avascular nature of the tissue (Nasiri et al. 2019). Understanding the basic science of AC structure and function can help scientists and clinicians to design considerable translational studies on cartilage repair and regeneration approaches.

1.2 Cartilage Tissue in Mammalian Body

Three distinct forms of cartilage can be recognized throughout the mammalian body depending on the composition of the matrix which include elastic cartilage, fibrocartilage, and hyaline cartilage, each of them having different structural, mechanical, and biochemical properties (Bhosale and Richardson 2008; Camarero et al. 2016).

The yellow cartilage present in the epiglottis, trachea, and ear lobes is elastic cartilage which support the shape and pliability of the organs (Camarero et al. 2016). The random orientation of elastin fibers provides high tissue elasticity (Mansfield

et al. 2009). Fibrocartilage is the hardest form of cartilage in the body that is found in the intervertebral disks, meniscus, pubic symphysis, and at the interface between ligaments and tendons with bone. Fibrocartilage has a high content of type I collagen and minor amounts of type II collagen, which together create a strong longitudinal network (Buchanan 2022). Hyalin cartilage is the most abundant cartilage type in the body which in the embryo is the base for bone formation (endochondral ossification) and in the adult body is present in trachea, costal cartilage, and in joints as articular cartilage. Articular cartilage covers the bone articular surfaces of the knees, shoulders, hips, and elbow joints (Brody 2015) and supports the load of body weight specially in the case of knee articular joint (Shrive et al. 1978).

1.3 Articular Cartilage

1.3.1 Origin of Articular Cartilage: Embryology of Cartilage

During the embryonic stage, three definite cell layers appear which are known as the ectoderm, mesoderm, and endoderm. Between the fourth to seventh weeks of gestation, the limbs originate from the mesoderm (Hall and Miyake 1992a). From the embryonic stage to skeletal maturity, cartilage tissue serves as a template for bone development, thereby before the onset of bone formation, the skeletal template in the early embryonic limb is made of a contiguous cartilaginous anlage with flattened, compressed, and homogenous mesenchymal cells which are named as “interzone” (as a sign of start of joint formation) at putative joint sites (Holder 1977; Mitrovic 1978). The removal of interzone from chick embryos inhibited limb joints formation over time (Holder 1977). During limb development and before the appearance of the interzone, interaction between mesenchymal stem cells (MSCs) and MSCs or substrate led to further cell mitosis and cell condensation. Within the condensate, the MSCs differentiate into pre-chondrocytes and form the cartilage anlage. Pre-chondrocytes then differentiate into mature chondrocytes that secrete extracellular matrix (ECM) and form hyalin cartilage, and proliferative chondrocytes which form the growth plate (Pacifi et al. 2006).

Soon after the appearance of the interzone, the joint site will be distinguished into a stiff “intermediate” and two surrounding “outer” compartments having more slackly arranged cells (Hyde et al. 2008). Between 8 and 12 weeks, proliferating cells within the growth plate promote blood vessel extension and facilitate the formation of bone collar and primary spongiosa at the first ossification center (Thompson et al. 1989). Primary spongiosa continues to elongate and forms trabecular bone as a result of enormous increase in chondrocyte volume (Kronenberg 2003). Intrinsic capacity for cell hypertrophy as well as cell swelling seems to be main reason for such change in chondrocyte volume (Bush et al. 2008).

A second ossification center appears at the other end of the cartilage anlage and encloses the cartilage in the interzone (Kronenberg 2003). Accordingly, these studies have determined that different joint tissues including articular cartilage have a heterogeneous origin, including de-differentiated chondrocytes and also cells

neighboring the cartilaginous anlagen (Decker 2017). Early cartilage cells within the interzone may increase the expression of Gdf5, Wnt9a, Wnt4, Erg, and Dcx during early joint development (Alsalameh et al. 2004; Hyde et al. 2007; Hartmann and Tabin 2001; Iwamoto et al. 2007; Später et al. 2006). Accordingly, despite the initial mesenchymal characteristics, the progenitor cells within the interzone undergo tissue-specific changes during the joint maturation journey. The expression profile of the interzone as well as its function and fate can be regulated by a large number of molecular effectors and regulators (Decker et al. 2014). Gdf5 is particularly believed to be effective in control of limb developmental malformation including absent cruciate ligament development and long bones (Harada et al. 2007; Storm et al. 1994). It has also been known that Gdf5 can promote cell adhesion at the early stages of limb development and chondrocyte proliferation at later stages (Francis-West et al. 1999). The genetic lineage tracing studies have progressed our conception of interzone cell origin and fate (Soeda et al. 2010; Zhang et al. 2011).

Mechanisms regulating synovial joint formation and cartilage maturation are not fully understood. It has been found that labeled chondrocyte progenitors at the embryonic stage produce only non-migratory posterity cells but not columns of vertical chondrocytes which is different from the preceding appositional templates (Decker 2017).

1.3.2 Postnatal Articular Cartilage Growth and Expansion

A transition cartilage at the embryonic stage has chondrocytes which experience proliferation followed by maturation and hypertrophy and finally undergo the endochondral bone formation process. A distinguished feature of articular cartilage is that it has a permanent structure that is functional throughout the whole life period, at least in healthy individuals (Decker et al. 2015).

After birth, the skeleton undergoes extensive growth and remodeling. In this regard, the length of the long bones increases and it widens significantly to adapt to the joint and ensure proper biomechanical function during postnatal life (Williams et al. 2008; Jadin et al. 2005; Hunziker et al. 2007). The preliminary cartilage of a newborn baby is highly cellular with small cells and little amount of ECM. These chondrocytes proliferate and secrete ECM components that form compressed packed cells resulting in dense and matrix-rich tissue in postnatal life and develop laterally to coat the expanding surface of the epiphyses (Decker et al. 2015). Upon maturation, AC undergoes mechanical and hydrostatic forces, to which it responds and changes by differing its composition and structure (Decker 2017). The matured AC has increased thickness and obtains radial (micro) and zonal (macro) structure (Camarero et al. 2016). Adult AC differs along the tissue depth upon chondrocyte shape and order, ECM composition, and its orientation.

At birth, the extracellular matrix of AC contains collagen fibrils with anisotropic orientation that undergoes subsequent changes in arrangement and composition to form mature AC with highly specialized characteristics (Hunziker et al. 2007; Clark et al. 1997; Youn et al. 2006).

However, there are substantial challenges with the homeostasis and regeneration of mature AC. This is mainly due to low cell density as well as the aneural and avascular nature of AC (Decker 2017). Accordingly, mature AC fails to perform tissue turnover and regenerate in case of possible injuries due to aging, tissue degenerating diseases, and trauma (Heinemeier et al. 2016). Nevertheless, several studies have proved the existence of cells with progenitor capacity in adult AC tissue (Candela et al. 2014a, b; Grogan et al. 2009; Williams et al. 2010) which can be further characterized and used for AC repair strategies.

Early studies proposed that increased AC thickness and growth are caused by a region with high proliferating cells subordinated to the articular surface (Mankin 1962). According to a study by Mankin (1963), postnatal cell proliferation is also continued within a deeper area nearby calcified cartilage, but is stopped in the sub-superficial region at subsequent stages of postnatal growth.

In subsequent studies, the presence of the superficial proliferation zone was confirmed more than the deeper region, suggesting the superficial area as a primary responsible zone for postnatal AC growth (Mankin 1963; Hayes et al. 2001; Archer et al. 1994). Future studies hypothesized that the proliferating cells within the superficial zone manage the lateral expansion of AC surface whereas vertical growth of cartilage is attributed to the deeper zone with more rapid proliferation (Hunziker et al. 2007). Recent advances in cell tracking techniques have revealed that there is only a single layer of proliferating cells at the AC superficial zone by birth (Kozhemyakina et al. 2015). Labeling the cells within this layer showed that their progenies were present throughout the total thickness of the mouse AC by 1 month after birth, suggesting that the early proliferating cell population in the superficial layer served as progenitors of the total thickness of AC chondrocyte and the base of appositional growth postnatally.

A mature AC has distinct organization (in the form of different zones or layers) mainly due to type II collagen distribution as well as shape and orientation of the chondrocytes (Wu et al. 2008) which will be discussed later under the title of articular cartilage ultrastructure.

The load-bearing capacity of AC is largely dependent on synovial fluid. Synovial fluid contains water, hyaluronan, and protein-rich plasma ultrafiltrate (Blewis et al. 2007). The composition, viscosity, and amount of this liquid support the healthy functioning of the AC.

1.4 Articular Cartilage Macrostructure

1.4.1 Cartilage Composition

The composition of AC varies mainly upon the cartilage developmental stage. Approximately, 1–5% of the total volume of adult AC is dedicated to chondrocytes, and the remaining 95–99% belongs to the extracellular matrix (ECM) (Camarero et al. 2016). The ECM contains water, fibers (collagen, elastin), proteoglycans, glycoproteins, and non-collagenous proteins as well as inorganic ions dissolved in

the water including potassium, calcium, and chloride (Lai et al. 1991; Linn and Sokoloff 1965; Mlynárik and Trattnig 2000). The water content of tissue changes depending on tissue depth, 80% at the superficial area to 65% in the calcified zone (Buckwalter and Mankin 1998). Water flow helps nutrition distribution through different depths of the tissue as well as supplying lubrication. In the following, we will discuss the special zonal structure of AC in more detail.

1.4.2 The Mature Cartilage Matrix Structure and Function

The load-bearing capacity of AC mainly arises from its ECM composition and orientation. ECM quantity and content are directly related to the chondrocytes which synthesize and maintain the components (Carballo et al. 2017). Along with AC development, chondrocytes produce a large amount of type II collagen (Eyre et al. 1988), whereas type XI collagen, as the most abundant collagen type in premature AC, disappears gradually due to the lagging suspension of cartilage to bone conversion (Camarero et al. 2016). Collagen accounts for about 75% of the cartilage dry weight and is the main load-carrying portion of the AC matrix. There is a concentration gradient of collagen microfibrils from the surficial areas to the depth of the cartilage such that it decreases by 20% in the calcified zone (Mow and Guo 2002). Collagen II organized in fibrils is the prevailing collagen type in AC, but AC is also comprised of collagen types III, IX, and XI. Collagen II provides AC with tensile strength and is the predominant collagen type in the pericellular matrix around the chondrocytes that can play an important role in cell signaling control and regulating chondrocyte fate and function (Wilusz et al. 2014). In a response to the mechanical forces loaded onto AC, the collagen fibers with random orientation align into new directions to increase the elasticity of the tissue (de Visser et al. 2008; Hunziker et al. 1997). Collagen glycosylation further elevates cartilage tightness (Bank et al. 1998).

Chondrocytes also synthesize proteoglycans as hydrophilic proteins which contain a protein core attached to glycosaminoglycans (GAGs) and have an important role in cartilage nutrition as well as water retention and efficient load attenuation (Brody 2015). The proteoglycan content varies along cartilage depth, and on average, it accounts for about 20–30% of the cartilage dry weight (Mow and Guo 2002). The GAGs consist of carbohydrate chains with repeating disaccharide units (Song et al. 2021). The major GAG subunits in AC are dermatan sulfate, chondroitin sulfate, and keratan sulfate. These negatively charged polysaccharide chains attach to water and positively charged ions including Na^+ and Ca^{2+} , thereby interacting with each other and making a strong network of fiber, water, and cells and supporting the mechanical properties of AC.

Hyaluronan is another component of the AC matrix which has a key role in proteoglycan functioning. The aggregation of proteoglycans is carried out via their attachment to hyaluronan by link proteins (Brody 2015). Aggrecan, the largest aggregating protein with up to 10,000 nm in length, is known as an aggregating complex resulting from attachment of multiple proteoglycans to a single hyaluronan

core. Aggrecan and collagen II establish AC resilience, as the basic biomechanical features of tissue. With aging, abnormal small aggrecans synthesized by aged chondrocytes can decrease AC strength (Brody 2015). Versican is another member of aggregating proteoglycans with a lower concentration. The mature AC matrix also contains decorin, biglycan, lumican, and fibromodulin as proteoglycans with small leucine-rich repeats (Gold and Beaulieu 2001), as well as lubrican and perlecan, two other types of proteoglycans (Gold et al. 2003; Guilak and Mow 2000; Hardingham and Bayliss 1990).

Proteoglycan aggregates along with the water trapped in their structure generate negative electrostatic repulsion forces and provide compressive elasticity to AC (Buckwalter 1998; Woo and Buckwalter 1988; Buckwalter and Mankin 1997). During joint loading, the local increase in interstitial fluid pressure inside the cartilage causes the water to flow out from the cartilage matrix and the induction of a great frictional stretch on the ECM. Once the joint load is removed, the water flows back into the AC matrix and preserves the continued function of the cartilage (Buckwalter and Mankin 1998; Buckwalter 1998; Buckwalter et al. 1994).

Various non-collagenous proteins and glycoproteins can be found in the AC matrix and based on their function; they can be divided into regulatory and structural subgroups (Frank and Grodzinsky 1987). Thrombospondin-1, thrombospondin-3, and thrombospondin-5, matrilin-1 and matrilin-3, tenascin-C, cartilage intermediate layer protein, and fibronectin are members of structural proteins. The category of regulatory proteins mainly includes chondromodulin-I and chondromodulin-II, bone morphogenic proteins, transforming growth factor- β (TGF- β), and cartilage-derived retinoic acid-sensitive protein (Sophia Fox et al. 2009). However, the specific function of some non-collagenous proteins and glycoproteins has not been fully characterized. Accordingly, AC is considered as a biphasic material in which collagen and other fibers in the AC matrix participate in load attenuation, as solids, whereas the proteoglycans and their attached water molecules decompose loads, as fluids (Brody 2015). Such supportive and interconnected function of ECM components indicates that the dysfunction of any members can put chemical and mechanical stress on the chondrocytes, causing disability to withstand compressive loads and AC dysfunction as a whole. In general, the functional role of the mature AC matrix can be summarized in several points: the matrix can protect the chondrocyte phenotype through mechanical support. AC matrix can preserve the signal transduction and nutrition diffusion into the chondrocyte. As a pool of growth factors and cytokines, the AC matrix is essential for cartilage cell growth and homeostasis (Mow et al. 1999; Xu et al. 2009; Brandl et al. 2010).

1.5 Microstructure of Articular Cartilage: Chondrocyte Structure and Function

Chondrocytes, as the only cell population present in cartilage and the origin of the majority of the cartilage matrix such as collagen fibers, appear to have a primary role in maintenance of cartilage function and health. The size, morphology, and location

of chondrocytes seem to be constant during decades of adult life (Buckwalter et al. 2005).

1.5.1 Chondrocyte Biology

Approximately, 1–2% of cartilage total volume is allocated to chondrocytes which are responsible for continuation of cartilage homeostasis through ECM synthesis and retention (Alford and Cole 2005). After skeletal maturity, the chondrocytes have limited proliferative capacity, despite their active metabolic function which enable them to respond to environmental signals (Hallett et al. 2019). Several studies suggest that oxidative stress along with telomere shortening may cause mitochondrial damage and chondrocyte inability in aged people (Ahmed et al. 2008; Yudoh et al. 2005).

Mature chondrocytes are specialized cells originating from mesenchymal stem cells. They are spherical in shape, metabolically active, and produce ECM components (Camarero et al. 2016). Any alteration in chondrocyte phenotype, count, and function could significantly affect AC health. Aging changes in the chondrocytes, including decreased cell density and cell size, could reduce the production of new fibers; thereby, the protective function of ECM is diminished and makes cells and other matrix components susceptible to further mechanical damage. Such a vicious cycle of chondrocyte inability and reduced fiber synthesis can cause progressive AC destruction (Brody 2015; Sophia Fox et al. 2009). Chondrocyte have rare cell–cell contact that is required for direct signal transduction, but they respond to environmental stimuli such as growth factors, hydrostatic pressure, and mechanical loads (Carroll et al. 2014). Chondrocytes receive nutrition through diffusion from the synovial fluid and survive in conditions with low oxygen concentration; therefore, they have anaerobic metabolism (Mankin 1982). After AC maturity, chondrocytes have a poor proliferation rate and low metabolic activity for several decades; therefore, the cell density of AC, as well as its regenerative responses, is significantly decreased.

1.6 Ultrastructure of Adult Articular Cartilage: Articular Cartilage Zone Organization

In the adult joint, when postnatal growth proceeds, AC shows a highly organized multi-zonal structure composed of superficial, medial, and deep zones, in which each layer has different chondrocyte shape and ECM structure (Carballo et al. 2017). AC zonation is fundamental for its long-lasting function and biomechanical behaviors (Hughes et al. 2005). The articulating surface of the joint is known as the superficial zone and is intended to resist forces. This layer contains small, elongated chondrocytes within a condensed ECM parallel to the AC surface. This layer enables the tissue to resist shear forces (Poole et al. 2001). Chondrocytes of the superficial zone are elongated and flattened in shape and have relatively silent metabolism

making regeneration difficult for this protective surface layer. They secrete collagen I, lubricating proteins such as hyaluronate and phospholipids which help to maintain frictionless joint movement (Jay et al. 2001). The appositional growth of AC also depends on the direction of chondrocyte proliferation in this layer.

The upper section of the superficial zone is known as the Lamina Splendas, the thickness of which in humans is confined to between a few hundred nanometers up to micrometers. Lamina Splendas has a non-cellular/non-fibrous structure suggesting that is comprised from the cumulative aggregation of synovial proteins (Jay et al. 2001). In addition, based on low proteoglycan concentration, the tissue permeability is greater than other AC parts (Bayliss and Ali 1978; Knudson and Knudson 2001). The precise role of the AC superficial zone has not been fully understood, but it serves as a preservative surface to decrease friction during load bearing and its architecture is most favorable to dispersing high shear force (Thambyah and Broom 2007).

The adjacent transitional/middle layer representing 40–60% of the total AC volume is composed of very larger and rounder chondrocytes with random organization. These cells within the transitional layer have more active subcellular organelles (i.e., mitochondria, Golgi apparatus, and endoplasmic reticula) and metabolic function, by which they secrete and deposit all of the typical hyaline cartilage ECM molecules including type II collagen (as the most prominent matrix component in the middle zone) and thus support tissue resistance to shear force as well as biomechanical resilience (Decker et al. 2015). The middle layer is also composed of large diameter fibers with random distribution. This structure suggests more repair capacity for this zone compared with the superficial layer.

At the bottom, the largest AC zone, known as the deep layer, the round chondrocytes are located perpendicular to the cartilage surface with column-like organization and are even larger in size with active metabolism leading to the production of matrix components (Decker et al. 2015). The highest proteoglycan content along with lowest water volume is one of the characteristics of the deep layer. The vertical organization in this layer, along with high proteoglycan content, is a favorable structure to resist tensile and compressive forces of the tissue (Brody 2015). Upon force loading, the total joint forces are comprised from the shear forces raised from the superficial zone as well as compressive and tensile forces from transitional and deeper layers (Bachrach et al. 1995).

The deep zone that represents about 30% of AC volume is under the middle layer and is characterized by low cell density, more glycoprotein, less collagen II content with the largest diameter and radial deposition, elongated chondrocytes organized in column orientation parallel to collagen fibers and the lowest water volume (Poole et al. 2001; Klein et al. 2009). Collagen X is also one of the ECM components in the deep zone. The deep zone and underlying calcified layer occupy 20–50% of AC thickness.

The calcified zone as a median between the subchondral bone and the superior cartilage links the AC to the underlying bone through anchoring the collagen of the deep layer bone matrix. The calcified layer has hydroxyapatite molecules which maintain physical stability and decrease the mechanical gradient. Rare chondrocyte

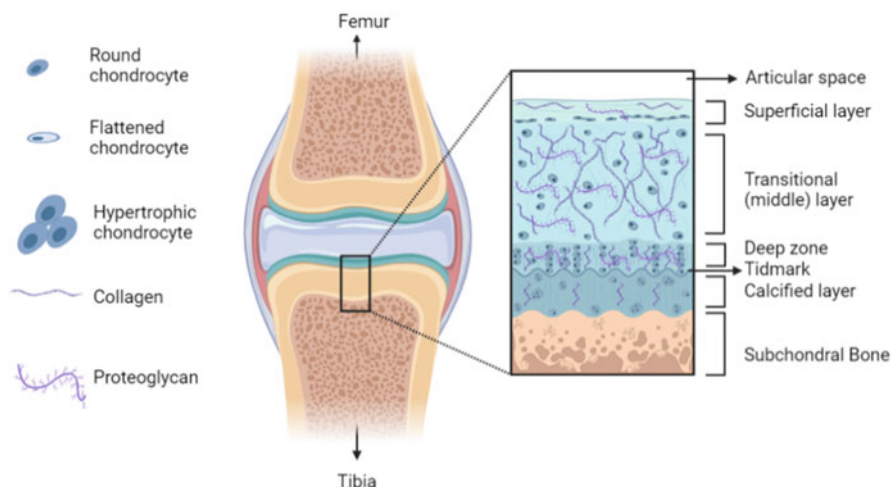


Fig. 1.1 Schematic drawing of different structural zonation in an articular cartilage full-thickness based on different composition and component organization

populations and a hypertrophic cell phenotype are other prominent characteristics of the calcified zone. A thin basophilic line which separates the calcified zone from the deep layer is the tidemark which provides an interface between mineralized and unmineralized areas (Carballo et al. 2017). The tidemark also separates the nutrition sources in pre-adulthood ages. In this time, diffusion from the synovial fluid supplies the nutrients to the tidemark above and below; nutrition is provided by the subchondral bone vasculature. In adults, bony vasculature is no longer able to supply nutrition, leaving the synovial fluid as the only nutrition source for adult AC, and limit its regeneration capacity (Brody 2015). Figure 1.1 represents the schematic concept of different structural AC zonations.

Structural layering of articular cartilage including superficial, transitional, and deep zone as well as calcified cartilage. The first layer is superficial zone which has flattened chondrocyte, high concentration of condensed collagen, and low content of proteoglycans. Transitional (middle) layer, as a thickest layer, has larger round shape chondrocytes and high amount of proteoglycan. In the third layer (deep zone), round chondrocytes are organized in columns and highest proteoglycan concentration belongs to this layer. The fourth layer is called the calcified layer, having large hypertrophic chondrocytes and hydroxyapatite molecules which maintain physical stability of AC. The visible border between uncalcified deep layer and underlying calcified zone is called tidemark.

1.6.1 Radial Structure (The Chondron)

In addition to zonal organization in AC structure, several distinguished regions are found in the AC matrix. These secondary microstructures which are recognized as

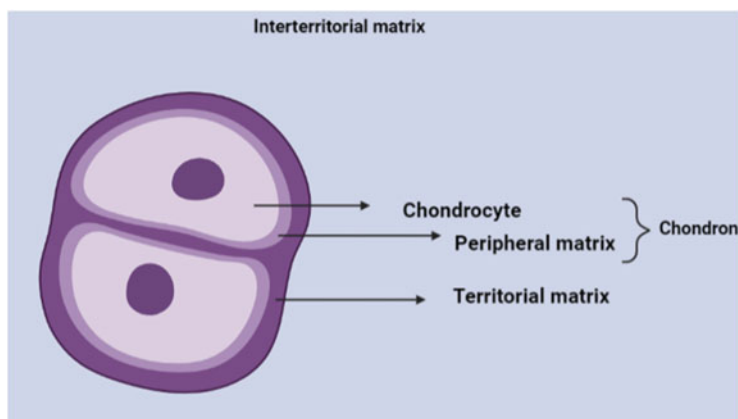


Fig. 1.2 Drawing illustration of the different layers of extracellular matrix surrounding articular cartilage cell (chondrocyte)

chondrons are formed based on the radial distance from the chondrocytes as well as the diameter and concentration of the matrix components including collagen II, X, VI fibrils, aggrecan, hyaluronan, and link proteins (Youn et al. 2006). Accordingly, the AC matrix can be divided into three functional regions, namely, the pericellular, territorial, and interterritorial regions. In this radially structured domain, the chondrocytes are surrounded by the pericellular matrix which are in turn encapsulated by the pericellular capsule. Chondron is also enclosed by the territorial matrix and the interterritorial matrix which generally form the ECM (Poole 1997) (Fig. 1.2).

In chondrocytes surrounding the pericellular matrix, the macromolecules appear to be dissociated since the complexes of hyaluronan–aggrecan are not completely formed. The macromolecule's size is also one of the differences between the pericellular and interterritorial matrix. For example, collagen II fibrils are smaller (10–15 nm) in the pericellular matrix and form an interwoven construction named the pericellular capsule (Poole et al. 1982). The chondron structure plays a key role in signal transduction initiation and provides a hydrodynamic defense for the chondrocyte upon weight bearing (Hall and Miyake 1992b).

The territorial matrix surrounding the pericellular matrix is a thicker region containing small collagen fibrils forming the interwoven network around the chondrocyte and has been suggested to provide the mechanical protection for chondrocytes. Around the territorial matrix, there is a larger region named the interterritorial matrix that mostly contributes to the AC biomechanical attributes. The interterritorial matrix has abundant proteoglycans as well as large collagen fibrils with random direction and parallel arrangement to the cartilage surface (Wilusz et al. 2014).

The matrix enclosed functional compartments representing the radial structure of articular cartilage which is called chondron. The chondron in turn is enclosed by territorial and interterritorial matrix, respectively.

1.7 Biomechanical Functions and Mechanical Characteristics of Articular Cartilage

A comprehensive definition for AC would be that AC is an exceptionally narrow layer of connective tissue which has a distinctive viscoelastic feature by which it can enable low friction joint movement and facilitate load transmission to the subchondral bone. AC can undergo high contact force and distribute comprehensive stresses to the underlying bone. In addition, AC facilitates the complex lubrication mechanisms during movement (Carballo et al. 2017).

AC is a biphasic medium in which water and inorganic ions form the fluid phase and the ECM component is considered as the solid phase. Negative electrostatic repulsion forces between the fluid and solid-phase ingredients produce compressive resilience in the AC (Maroudas and Bullough 1968).

When weight is applied, the trapped fluid is under pressure and flows out of the cartilage, this puts frictional drag on the cartilage matrix as a solid phase, then the weight is transferred (Soltz and Ateshian 1998; Cohen et al. 1998). Accordingly, the tensile, compressive, and shear reaction of AC originate from these two phenomena: its biphasic nature and the viscoelastic capacity of cartilage tissue. The viscoelastic capacity of cartilage also originates from two types of mechanisms: the frictional drag associated with the fluid phase (Mow et al. 1980; Simon et al. 1984), and the intrinsic viscoelastic action of the solid phase (collagen, glycoprotein) (Ahsan and Sah 1999). Figure 1.3 represents the important function of articular function.

Illustration demonstrating several biomechanical functions of AC. AC provides a low friction gliding surface, can absorb shock during load bearing, and reduces the

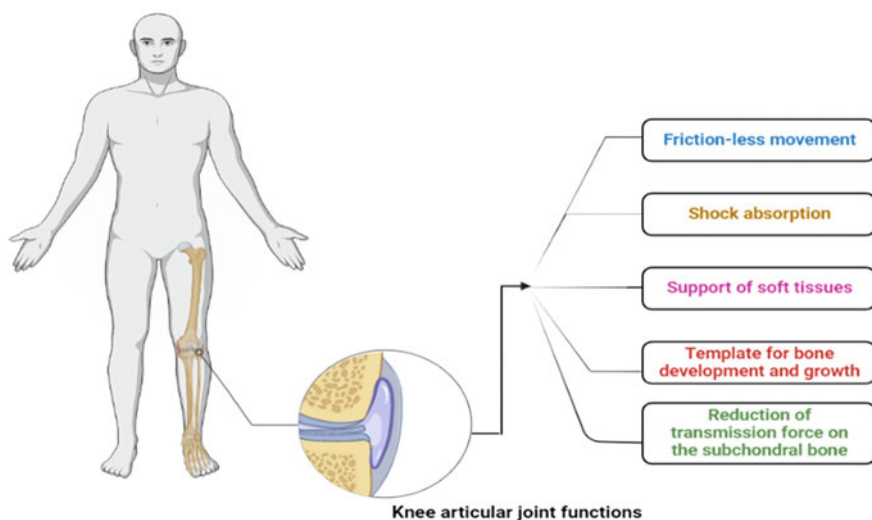


Fig. 1.3 Articular cartilage biomechanical functions

transmission force on the subchondral bone. Also, AC is essential for bone development and growth.

As a result, AC presents a low friction gliding surface, absorbs shock during load bearing, and reduces the transmission force on the subchondral bone (Bhosale and Richardson 2008). We can categorize the three major properties of AC as follows:

1.7.1 Compressive Properties of Articular Cartilage

The compressive property of AC originates from both the viscoelasticity and permeability of the cartilage tissue. AC response to applied mechanical force is nonlinear due to its viscoelastic and anisotropic nature. The pressurization rate of the interstitial fluid of cartilage is high because of the low permeability of the tissue; therefore, once a weight load is applied, the fluid starts to flow out of the cartilage producing a great drag force on the solid phase and minimizes the stress (Camarero et al. 2016). Accordingly, the AC response to the load is elevating the fluid-phase pressure as well as mechanical stiffening.

1.7.2 Tensile and Shear Properties

On the cartilage surface, tensile stress is generated in response to loading-mediated cartilage deformation. The solid-phase components including proteoglycan and collagen fibrils support tensile force which can cause collagen movement and tissue viscoelastic response. The solid phase including collagen fibrils and GAGs can regulate the tensile properties of AC. Accordingly, the tensile properties of AC are strongly dependent on tissue depth, orientation, and density of collagen (Eleswarapu et al. 2011).

1.7.3 Swelling Behavior of Articular Cartilage

When cartilage is damaged, swelling occurs at the early stages. In such situations, the average water content of the tissue increase up to 90% (Jaffe et al. 1974). Increased water content can greatly affect AC permeability and compressive stiffness (Mow et al. 1992). Such tissue capacity alteration can decrease the AC load-bearing capability, tissue functionality, and health.

As a dynamic reservoir, the synovial fluid also plays an essential role in AC function, nutrition, and homeostasis (Carballo et al. 2017). AC is an avascular tissue, so it receives nutritional molecules as well as proteins produced by synovial tissue via the synovial fluid. Accordingly, the synovial fluid composition can reflect AC conditions. Synovial composition also can provide efficient boundary friction for AC. The main synovial fluid ingredients to minimizing the friction of AC surface are phospholipids, lubricin protein, and hyaluronic acid (Perman 1980).

1.8 Metabolism of Articular Cartilage

The biochemical and biomechanical microenvironment surrounding the chondrocytes can change their metabolic activity and hemostasis. For example, the concentration of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-alpha) and interleukin-1 beta (IL-1b) has been shown to be associated with some catabolic and anabolic events resulting in cartilage degradation and synthesis (Buckwalter and Mankin 1998). Several regulatory peptides and growth factors including transforming growth factors and insulin-like growth factors have been found to be effective on proteoglycan metabolism, although its exact mechanism has not been fully understood yet (Sophia Fox et al. 2009).

Keeping up healthy AC metabolism mainly depends on active load and orderly joint movement, so that joint inactivity can lead to cartilage degradation (Buckwalter and Mankin 1998). AC matrix has a long half-life, estimated to be 100–400 years for collagen and 25 years for proteoglycans, for example (Masuda et al. 2003). Several enzymes including cartilage metalloproteinase, collagenase, stromelysin, gelatinase, cathepsin B, and cathepsin D are involved in AC degradation and turnover. AC catabolic events and its intrinsic degradation process are related to interaction between matrix metalloproteinase enzymes and cytokines (Carballo et al. 2017).

1.9 Maintaining a Healthy State: Articular Injuries and Disease

AC disorders can represent some debilitating conditions including aging, trauma, and orthopedic diseases. AC function has a direct relation with its composition. As age progresses, chondrocyte numbers remain unchanged but their distribution alters throughout different zones. In addition, the size and concentration of chondrocyte-synthesized ECM components including proteoglycans and collagen change with increasing age (Martin and Buckwalter 2001) which can lead to AC injuries and physical disability of the joint.

Typically, a tissue responds to injuries through a sequence of healing processes such as necrosis, inflammation, repair, and scar remodeling events, by which tissue vascularity plays an important role in the formation of a normal response. As an avascular tissue, AC lacks the capacity for a normal healing response, leaving its reparative ability at very low levels.

AC injuries can be divided into two general types: (1) direct mechanical defects of the cartilage matrix without chondrocyte damage. In such conditions, the healthy chondrocyte can synthesize new proteoglycans and restore cartilage function. (2) Mechanical defects of both the chondrocyte and matrix components in which the tissue regenerative response depends on several factors including the size and depth of the defect, patient age, and the genetic predisposition of the patient (Bhosale and Richardson 2008).

1.10 Conclusion

This brief review summarizes the present knowledge about the development and growth of articular cartilage as a highly specialized form of connective tissue, covering the articulating surface of synovial joints. However, more details still remain uncovered. Our conception of AC origin from the interzone mainly comes from the genetic lineage tracing studies which have determined that the cartilage progenitors result from the differentiation of MSCs as well as surrounding cells of cartilaginous anlage. The principal function of postnatal mature AC is to provide frictionless movement by forming a lubricated surface for articulation and helping to transmit loads through interaction between the fluid and solid phases of tissue. An exact understanding of AC composition and structure makes a promising future for its challenging regenerative medicine.

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Cartilage Defects and Diseases: Conventional Therapies and Its Limitations

2

Xinwei Wang and Peng Xia

Abstract

Articular cartilage has a supportive, cushioning, and lubricating role. Damaged cartilage not only stimulates an intra-articular inflammatory response, but also leads to an imbalance in the biological force lines, causing pain, deformity, and motor dysfunction. Different types of cartilage defects and diseases have different pathogenesis and treatment strategies. This article mainly summarizes the conventional therapies for osteoarthritis, rheumatoid arthritis, and gouty arthritis and their limitations.

Keywords

Arthritis disease · Cartilage · Therapy · Limitation

2.1 Introduction

Trauma, obesity, and autoimmune diseases can cause different degrees of joint disease. The main pathogenesis of joint disease is cartilage defect, inflammatory infiltration, and metabolic microenvironment imbalance (Salucci et al. 2022). Cartilage tissue plays a role in supporting, buffering, and lubricating the human body during exercise. Cartilage damage will not only stimulate inflammatory responses, but also lead to the imbalance of biological forces in the joints, affecting multiple joint pain, deformity and dysfunction, and even leading to disability (Mata-Miranda et al. 2016). Cartilage lacks blood supply and nerves and mainly relies on synovial fluid in the joint to provide nutritional needs, which also results in poor self-repairing

X. Wang · P. Xia (✉)

Department of Rehabilitation Medicine, Nanjing First Hospital, Nanjing Medical University, Nanjing, Jiangsu, China

ability of cartilage (Tsang et al. 2015; Du et al. 2022). Therefore, cartilage defects and cartilage repair are great challenges in medicine today.

Articular cartilage defects can be classified as osteoarthritis (OA), rheumatoid arthritis (RA), and gouty arthritis (GA) according to the location of the cartilage defect and whether it is accompanied by inflammation. OA is a degenerative disease of the articular cartilage and is likely to occur in joints that are heavily weight-bearing. RA usually involves several joints such as interphalangeal joints, wrist joints, ankle joints, and knee joints and is characterized by non-specific inflammation of peripheral joints. GA is most often seen in the metatarsophalangeal joint of the bunion, but can also occur in other larger joints, especially in the ankle and foot joints, and is usually accompanied by acute inflammatory episodes.

2.2 OA

OA is a chronic joint disease characterized by articular cartilage degeneration and secondary bone hyperplasia. The clinical manifestations are progressive joint pain, which may include morning stiffness and joint swelling, which seriously affects the quality of life of patients and even leads to disability. The incidence of OA is increasing year by year, and it has exceeded 7% (Leifer et al. 2022). At present, the treatment of OA mainly includes drug therapy, non-drug therapy, cell therapy, and surgical treatment (Fig. 2.1).

2.2.1 Progress and Limitations of Drug Therapy for OA

The drug therapy of OA includes oral anti-inflammatory analgesics, intra-articular injection drugs, and chondroprotective agents. Nonsteroidal anti-inflammatory drugs (NSAIDs) are conventional drugs for the treatment of OA. By reducing the production of inflammatory mediator prostaglandins, they have anti-inflammatory, analgesic, and antipyretic effects. However, long-term use of NSAIDs can also cause digestive system damage (Hochberg et al. 2021). Therefore, it is not recommended as a first-line drug for the treatment of OA. Hyperalgesia occurs in 15–40% of OA patients (Roby et al. 2022) and is resistant to treatment drugs. Even mild stimulation can cause severe pain with symptoms such as insomnia, fatigue, anxiety, and depression (Fingleton et al. 2015). These patients are advised to take opioids and anti-anxiety medications, which can significantly relieve symptoms (Lluch Gírbés et al. 2013). Topical medicines or Chinese patent medicine patches can play an anti-inflammatory and analgesic effect, but occasionally there may be local skin rash, burning sensation, itching, and other adverse reactions (Tanabe et al. 2021).

The effect of intra-articular injection of sodium hyaluronate can last for more than 12 weeks, which is safer than repeated intra-articular injection of glucocorticoids (Kim et al. 2021). However, some studies have shown that there is little difference in the effectiveness of intra-articular hyaluronic acid injection and 0.9% sodium chloride injection solution injection (Turajane et al. 2007). Currently, there are

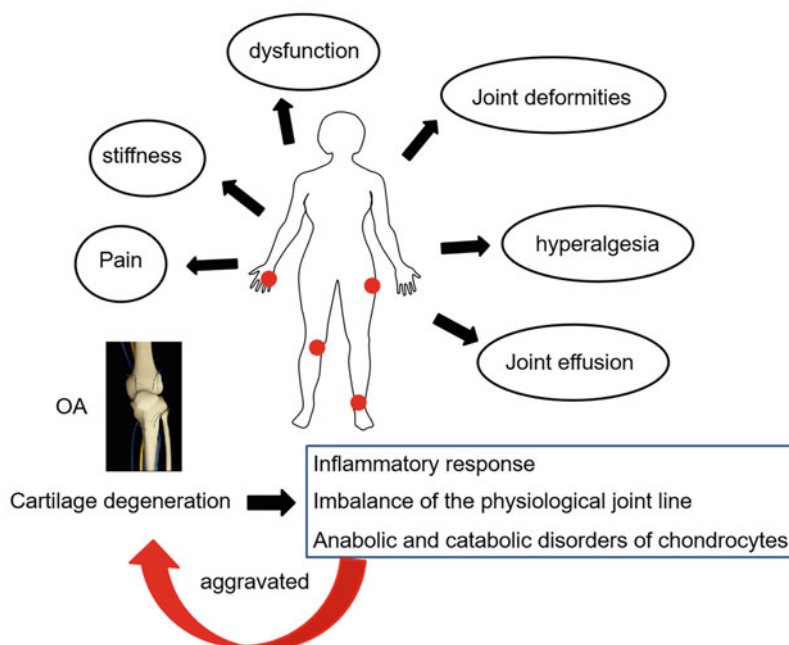


Fig. 2.1 Clinical manifestations and pathological mechanisms of osteoarthritis (OA). OA commonly occurs in large joints such as the knee, hip, and ankle. The main manifestations are pain, morning stiffness, dysfunction, and joint swelling. The pathology of OA is the degeneration of articular cartilage, which causes inflammation and metabolic abnormalities, which in turn aggravate the degree of cartilage degeneration

differences in the recommended direction and strength of different OA guidelines for intra-articular injection of hyaluronic acid. For example, the American Association of Orthopaedic Physicians (AAOs) guidelines do not recommend intra-articular injection of hyaluronic acid (Kolasinski et al. 2020), and 2014 International Osteoarthritis Society (OARSI) guidelines are weak recommendations (Bannuru et al. 2019).

Glucosamine sulfate (GAS) is an anti-inflammatory and chondroprotective drug widely used in OA. It can stimulate the synthesis of proteoglycan and collagen in the extracellular matrix of cartilage and inhibit the secretion of pro-inflammatory factors (Rovati et al. 2012; Tenti et al. 2022). The oral bioavailability of GAS is only about 10%, and the therapeutic effect is not significant. When it is directly used in the joint cavity, there are also problems such as high local concentration and short residence time (Agiba et al. 2018). Recently, some studies have proposed the use of hydrogenated phosphatidylcholine to construct GAS-encapsulating liposomes (GAS-lips), which avoids the disadvantages of low oral availability and short residence time for local injection. GAS-lips release GAS slowly and continuously in the joint cavity and have a certain lubricity, which can delay joint wear and play a

therapeutic role, and provide a research basis for the development of osteoarthritis nanopreparations (Ji et al. 2022).

2.2.2 Progress and Limitations of Non-drug Therapy for OA

Non-drug treatments include physical factor therapy, exercise therapy, and bio-mechanical interventions. Physical factors commonly used in the treatment of OA include electrotherapy, phototherapy, magnetic therapy, heat therapy, wax therapy, mud bath therapy, and ultrasonic therapy. The commonly used electrotherapy in clinic includes low-, medium-, and high-frequency electrotherapy. These three types of electrotherapy can heat the body tissue evenly from shallow to deep, so as to speed up the body's absorption of inflammatory exudates and the discharge of metabolites. The electrotherapy also can stimulate the repair of connective tissue and effectively relieve muscle spasm, relieve joint pain, and other symptoms. It can also improve local tissue microcirculation, increase vascular permeability, promote fracture repair and healing, and prevent cartilage degradation (Kaya Mutlu et al. 2018). The main effect of phototherapy is the thermal effect, which can increase the temperature of local tissues in the human body, thereby expanding local blood vessels, enhancing metabolism by accelerating blood circulation, improving the immune function of the body, anti-inflammatory and analgesic, and promoting cartilage regeneration (Makolinets et al. 2019; Alfredo et al. 2022). Some scholars believe that phototherapy can exert anti-inflammatory effects by affecting the generation of osteoblasts and the synthesis of collagen, stimulate angiogenesis, rebuild the blood supply of subchondral bone, and increase blood circulation (Liao et al. 2020). Magnetic therapy is an alternating electromagnetic field generated by pulsed current in the coil. When applied to the human body, magnetic field can expand local blood vessels, promote blood circulation, weaken the activity of pain-causing substances by reducing blood viscosity, improve hemorheological properties, and thus relieve the pain of OA patients (Karateev et al. 2021). Hyperthermia, wax therapy, and mud bath therapy mainly improve blood circulation through warming, promote the infiltration and absorption of OA inflammation, relieve spasm, and then achieve the purpose of analgesia (Ho et al. 2021; Harada et al. 2021). A large number of studies have shown that ultrasonic therapy can reduce inflammation, edema, improve pain, and promote the repair of articular cartilage in OA patients (Jo et al. 2022; Xia et al. 2015, 2022). However, the current physical therapy lacks a unified parameter standard, which is not conducive to the long-term clinical research of OA.

2.2.3 Progress and Limitations of Exercise Therapy for OA

From the perspective of the occurrence and development of OA, obesity, bone and muscle damage, and functional muscle decline are usually the main intervention targets for disease prevention. Exercise training can effectively promote the growth of muscle fiber volume, thereby increasing muscle strength and slowing muscle

decline. Therefore, exercise is an effective measure to prevent OA (Grimm et al. 2015). A number of guidelines recommend that water sports, cycling, aerobic walking, stretching, and other sports are the main daily exercise methods for OA patients (Zjuan et al. 2022; Chen and Yu 2020; Kloppenburg et al. 2019). Long-term activities such as running, jumping or climbing, and climbing stairs increase the risk of joint wear and tear (von Heideken et al. 2021). In addition, functional exercise can maintain joint range of motion, improve muscle strength, increase balance and coordination function, effectively alleviate the progression of OA cartilage damage, and prevent accidents such as falls (Iversen 2010; Khalaj et al. 2014). A large number of studies have shown that exercise can reduce local inflammatory factors in the OA joint, reduce the inflammatory infiltration of pain neuron fibers, block the pain conduction pathway, and delay the process of OA cartilage degeneration (O'Neil et al. 2018). Recently, studies have also demonstrated that high-intensity intermittent exercise is more effective than moderate-intensity continuous exercise in relieving pain in OA patients, especially the pain sensitization caused by OA (Hartley et al. 2020; Keogh et al. 2018). The course of exercise is generally recommended to last for 8–12 weeks, 3–5 times a week, and 30–45 mins of exercise will bring the greatest advantage to the treatment of OA. No matter what exercise method is chosen, rehabilitation physicians should make exercise prescriptions individually based on the age, gender, course of disease, disease condition, and exercise basis of OA patients and should follow up and guide regularly. For patients, planned and guided training can improve the compliance of OA patients and can also significantly improve the pain and dysfunction of patients (Shavazipour et al. 2022; Santos-Moreno et al. 2020).

Poor alignment of lower extremities is an important risk factor for cartilage damage in OA. Uneven joint force or excessive joint stress will cause imbalance in cartilage tissue decomposition and repair, leading to joint deformity (Shakoor et al. 2012). Braces and orthoses can change the biomechanical situation of the joint. Wedge-shaped insole and valgus knee brace can be used to combat the uneven force on the medial side of the knee joint, reduce pain, and improve joint function (Yılmaz et al. 2016). The vast majority of clinical guidelines recommend OA patients using walking auxiliary equipment, such as walking sticks as part of the knee OA patients comprehensive conservative strategy, and without any auxiliary equipment, compared to the contralateral use cane to assist walking, an average of about 7–10% of knee adduction torque can effectively prevent and delay the lower extremities deformity (Reeves and Bowling 2011).

2.2.4 Advances and Limitations of Cell Preparations and Stem Cell Therapy for OA

In recent years, cell preparation and stem cell therapy are the hot spots of research and also the new trend of OA treatment. Cell preparation has good anti-inflammatory, analgesic, cartilage repair, and cartilage regeneration effects, so as to delay the cartilage damage and improve joint function of OA. Platelet-rich plasma

(PRP) and platelet lysate (PL) are common preparations for the clinical treatment of OA (Dhillon et al. 2019). The concentration of platelets in PRP is an important factor in the treatment effect of OA, and the internationally recognized effective concentration is $1000 \times 10^9/\text{L}$. Both autologous and allogeneic purified PRP can improve the proliferation ability of OA chondrocytes and promote cartilage repair, but autologous PRP has more obvious therapeutic effect (Su et al. 2022; Filardo et al. 2021). In 2010, Sampson et al. (2010) demonstrated for the first time that intra-articular injection of PRP can reduce pain and improve joint function in patients with OA, and the mechanism may be related to peripheral blood pain mediator sensory neuropeptide substance P, serotonin, prostaglandin E2 (PGE2), interleukin-1 (IL-1), and tumor necrosis factor α (TGF- α). However, whether PRP preparation preserves leukocytes or activates platelets is still controversial (de Castro et al. 2022), and whether platelet activation before PRP treatment has not been reported on the therapeutic effect. In addition, residual red blood cells in PRP will disintegrate hemolysis and promote inflammation, affecting the therapeutic effect of OA (Everts et al. 2019).

Intranuclear mononuclear cells (MNCs) are cells with a single nucleus. They consist of heterogeneous cell populations such as stem cells and progenitor cells. At present, the clinical application of bone marrow mononuclear cells (BM-MNCs) and adipose tissue MNCs in the treatment of OA has been studied (Tawfeek and Esaily 2022). Clinical studies have found that intraluminal injection of MNCs can inhibit the expression of pro-inflammatory and catabolic molecules, achieve anti-inflammatory, analgesic, repair articular cartilage damage, and promote cartilage regeneration, and its therapeutic effect can be maintained for 2–5 years (Goncars et al. 2019). However, intra-articular injection of MNCs can also cause complications such as joint swelling, pain, and reactive effusion.

Intra-articular injection of mesenchymal stem cells (MSCs) is the frontier and hot spot in the treatment of OA (Lee et al. 2021). Umbilical cord blood MSCs (Cartistem) have been approved and marketed, and 13 new stem cell drugs for the treatment of OA have entered clinical trials. Dilogo et al. (2020) found that the pain score of OA patients was significantly reduced, and the effect was significantly better than that of hyaluronic acid in a randomized controlled trial of cord blood MSCs in the treatment of knee OA. Although some patients had transient joint effusion and pain, they all improved after rest or oral acetaminophen (Ibáñez et al. 2022). It is generally believed that there is no significant difference in the effects of different sources of MSCs in the treatment of OA. Low dose of MSCs in the treatment of early OA has a good analgesic effect, while high dose will cause local pain and swelling, but has a good effect in repairing cartilage damage and promoting cartilage regeneration (Shoukrie et al. 2022). There was no significant difference in clinical efficacy between single and multiple injections of MSCs, but multiple injections could delay the progression of cartilage defect. MSCs therapy mainly acts through paracrine, and MSCs exosomes (MSCS-EXOs) are an important substance in the paracrine effect of MSCs (Xu and Xu 2021). At present, the research of exosomes in the treatment of OA mainly focuses on MSCS-EXOs, embryonic stem cell-derived MSCs, induced pluripotent stem cell-derived MSCs, human bone marrow MSCs, adipose MSCs,

amniotic MSCs, and umbilical cord MSCs (Wang et al. 2022a; Yokota et al. 2019). Intra-articular injection of MSCS-EXOs has anti-inflammatory effects, slows down the degradation of chondrocyte extracellular matrix, reduces chondrocyte apoptosis, promotes the repair and regeneration of injured cartilage, and delays the progression of disease (Zhou et al. 2020). Although there are many studies on the treatment of OA by intra-articular injection of cell preparations, most of them are single-center studies with short follow-up time, small sample size, few randomized controlled trials, and no unified standard for injection dose, frequency, and observation indexes, which have adverse effects on the evaluation of cell therapy (Mohd Noor et al. 2021).

2.2.5 Progress and Limitations of Surgical Treatment of OA

OA patients will have different degrees of cartilage damage in the late stage, accompanied by hyperosteoplasia, synovitis, deformities, etc., which will generally be treated by surgery, including arthroscopic debridement, osteotomy, and joint replacement (Gong et al. 2018). Although there are a variety of surgical methods for OA complicated with extraarticular deformities, the optimal surgical plan has not been determined at present, which is whether extraarticular osteotomy and total knee arthroplasty should be performed simultaneously or in stages. If it is performed by stages, will the order of external osteotomy correction and total knee arthroplasty affect the postoperative effect? If the operation is performed at the same time, the complications of intra-articular infection and partial bone nonunion of osteotomy will increase (Teoh et al. 2017; Rahmansyah et al. 2022). With the application of computer-based intelligent devices in the field of orthopedics, orthopedic surgery is bound to be diversified, but the best surgical plan still needs to be combined with the comprehensive and complete clinical evaluation and imaging examination of OA patients, so as to select the most suitable surgical treatment for patients (Fig. 2.2).

2.3 RA

RA is an autoimmune inflammatory disease with an incidence rate of 0.5–1% (Lau et al. 2015). It usually involves multiple joints such as interphalangeal joint, wrist joint, ankle joint, and knee joint, and its clinical manifestations are joint redness, pain, stiffness, and even disabling complications such as joint rigidity and deformity (Ben Mrid et al. 2022). According to the American College of Rheumatology (ACR), the European League Against Rheumatism, EULAR) and the “treat-to-target” principle recommended by the Asia Pacific Rheumatoid Association, the treatment of RA requires maximal relief of symptoms, maintenance or restoration of normal body function, and prevention of joint damage (Wells et al. 2009; Ellrodt et al. 2022; Gaujoux-Viala and Gossec 2014).

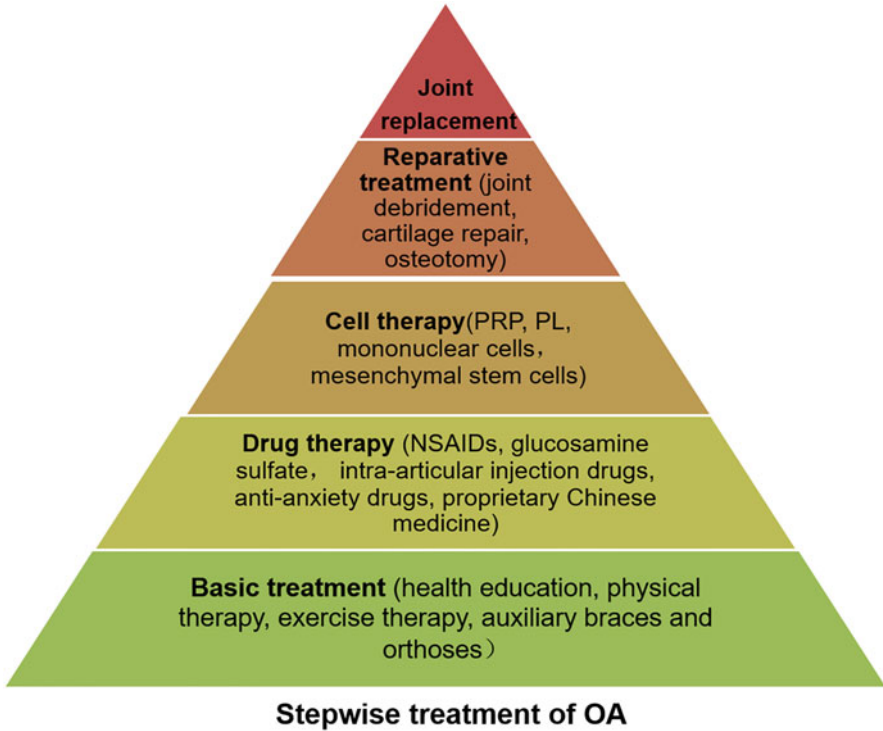


Fig. 2.2 Stepwise treatment strategies for osteoarthritis (OA). The treatment of OA mainly includes basic treatment, drug therapy, cell therapy, reparative treatment, and joint replacement

2.3.1 Progress and Limitations of Drug Therapy for RA

RA is mainly treated with drugs that relieve symptoms and delay the progression of the disease. Non-steroid anti-inflammatory drugs (NSAIDs) mainly prevent the production of prostaglandins, prostacyclin, and thromboxane by inhibiting cyclooxygenase to improve pain (Wang et al. 2022b). However, massive and long-term use of NSAIDs may also lead to some side effects, such as nausea, vomiting, hearing impairment, gastrointestinal ulcers, or bleeding (Lin et al. 2022).

Glucocorticoids can rapidly alleviate the clinical symptoms and reduce inflammation of RA by reducing phospholipid release and inhibiting eosinophil activity. However, long-term or massive use of hormones is faced with greater side effects, including osteonecrosis, osteoporosis, abnormal glucose metabolism, and immunosuppression (Ferreira et al. 2016). So NSAIDs and corticosteroids are recommended for rapid control of disease onset or progression in a short period of time.

Disease-modified anti-rheumatoid drugs (DMARDs) reduce the number of inflammatory cells by interfering with DNA synthesis by competitive inhibition of dihydrofolate reductase. It can effectively slow down joint destruction and disease progression and also reduce the incidence of RA complications. Currently

commonly used DMARDs are methotrexate, hydroxychloroquine, sulfasalazine, etc. (Harrold et al. 2016; Cordisco et al. 2022). However, the effect of DMARDs is slower than that of corticosteroids. Long-term application of DMARDs can also cause serious side effects such as gastrointestinal reactions, liver damage, and bone marrow suppression. Therefore, RA patients often need regular follow-up in order to detect early adverse reactions in time (Syngle et al. 2017).

2.3.2 Progress and Limitations of Biologic Therapy for RA

In recent years, the emergence of biological DMARDs (bDMARDs) and targeted synthetic DMARDs has injected new vitality into the treatment of RA (Bastida et al. 2017). These drugs mainly target key factors or cells in RA inflammation, block inflammatory pathways, and can quickly and effectively slow down the progression of RA (Mori 2020). At present, the most widely used bDMARDs are TNF- α inhibitors, IL-1 blocking drugs, and Janus-activated kinase (JAK) inhibitor drugs, which can effectively prevent the recruitment of pro-inflammatory cells and the release of downstream factors, thereby rapidly relieving symptoms (Poiroux et al. 2015; Liu et al. 2020). However, long-term application of bDMARDs may face risks such as immune suppression, infection, tuberculosis, and the occurrence of malignant tumors (Takeuchi et al. 2018). The optimization of traditional drugs, the development of new drugs, and the improvement of half-life and bioavailability of drugs can improve the treatment effect and compliance of patients.

2.3.3 Progress and Limitations of Exercise Therapy in RA

More and more studies have shown that regular exercise in patients with RA is helpful to maintain or increase joint flexibility, improve function, reduce pain, and reduce disability rate (Liao et al. 2022; Modarresi Chahardehi et al. 2022). Exercise intervention mainly includes aerobic exercise, strength training, combination of aerobic exercise and strength training, joint gymnastics, and Tai Chi. Moderate aerobic exercise (when the heart rate reaches 60–80% of the maximum heart rate during exercise) can not only make the muscles relax and alleviate the spasm of the tissues around the joint, but also facilitate the blood circulation of the local joint, prevent the accumulation of inflammatory substances, and promote the dissipation of inflammation (Azeez et al. 2020). Aerobic exercise can not only improve the physiological function of patients, but also improve their psychological and social functions and health self-awareness, thus improving the overall quality of life of patients (Ye et al. 2022). Ye et al. conducted a meta-analysis on the application effect of aerobic exercise in patients with RA, and the results showed that aerobic exercise could relieve joint pain, reduce disability rate, and improve quality of life (Ye et al. 2022). In addition, the combination of aerobic exercise and strength training can not only improve the cardiopulmonary function of patients, reduce the incidence of cardiovascular diseases within 10 years, but also improve muscle strength (Dos

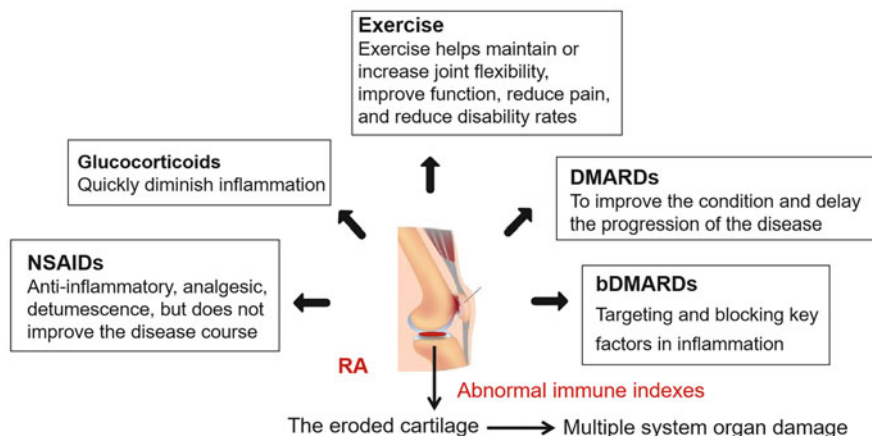


Fig. 2.3 Treatment strategies for rheumatoid arthritis (RA). The treatment of OA mainly includes non-steroid anti-inflammatory drugs (NSAIDs), glucocorticoids, disease-modified anti-rheumatoid drugs (DMARDs), biological DMARDs, and exercise

Santos et al. 2021). The study of Strasser et al. showed that after 6 months of strength training combined with exercise, not only could significantly improve the cardiopulmonary function (10%) and muscle strength (14%) of RA patients, but also could relieve pain and reduce disease activity in the long term (Strasser et al. 2011).

Joint gymnastics can enable each joint to maintain its own range of motion and prevent RA disease from damaging the joint and affecting the daily living ability of patients (Srikesavan et al. 2020). More than 90% of RA patients first involve the wrist joint, so wrist joint functional exercise plays an important role in the rehabilitation of RA patients (Brorsson et al. 2009). Jing et al. designed the wrist joint function exercise for 15–30 min each time, twice a day (after morning and nap), for 12 weeks. After 12-week exercise, the duration of morning stiffness, joint tenderness, and joint swelling in the experimental group were better than those in the control group (Jing et al. 2014). This indicates that the wrist joint gymnastics plays an important role in reducing joint contracture deformation in patients with RA. RA is not only easy to invade the wrist joint, but also often involves other joints in the whole body. Therefore, systemic joint exercise should also be paid attention to.

Tai Chi exercise has certain positive effects on the physiology and psychology of RA patients. The 14th section of Tai Chi bimodal perforation helps prevent elbow stiffness and upper limb muscle atrophy, osteoporosis. The 19th section needle in the haystack helps to maintain or enhance quadriceps muscle strength and mobility, prevent and correct knee deformity, and improve self-care ability. Tai Chi exercise can not only reduce joint swelling, improve lower limb muscle strength and endurance, but also improve patients' perceived level of their own physical condition (Lee et al. 2012; Francesco 2021; Mudano et al. 2019) (Fig. 2.3).

2.4 GA

GA is a common type of metabolic joint inflammation, with an incidence of 2.7–6.7%, and the incidence of GA in young men is about twice that of women. The occurrence of GA is mainly related to the deposition of monosodium urate (MSU) crystals in the joint cavity (Bursill et al. 2019), and MSU leads to cartilage damage of GA by reducing the viability of chondrocytes and promoting catabolic state (Chhana et al. 2013). Acute GA usually presents with severe joint pain, accompanied by signs of redness, swelling, fever, and dysfunction. With the deposition of MSU, chronic GA gradually leads to joint structural destruction, resulting in joint pain, deformity, and dysfunction (Schlesinger 2004). Different from OA and RA, acute GA is a self-limited disease with a course of 7–10 days, which is related to the anti-inflammatory mechanism of the body's autoimmunity (Sunkureddi 2011). Recurrent GA will form gout nodules in the affected joints, and the formation of gout nodules may be an “inflammation, resolution and tissue remodeling” process of the body against MSU, which will destroy the bone and cartilage of the joint (Shi et al. 2021).

2.4.1 Progress and Limitations of Drug Therapy for GA

The key to GA treatment is urate-lowering therapy (ULT), which mainly includes allopurinol and febuxostat (Tausche and Aringer 2016). In addition to ULT, NSAIDs, colchicine, and corticosteroids are also used in the treatment of acute GA (Towiwat et al. 2020). NSAIDs mainly inhibit the synthesis of prostaglandin by inhibiting cyclooxygenase to control the symptoms of GA, such as redness, swelling, heat, and pain, commonly used are puzen, indomethacin, and etoricoxib. NSAIDs have a rapid anti-inflammatory effect, and there is no obvious difference between the anti-inflammatory effects of various NSAID drugs. However, long-term or high-dose application may cause nausea, gastrointestinal bleeding, and other complications (Wan Ghazali et al. 2021). For people with reduced renal function, heart failure and other diseases need to be carefully considered.

Colchicine can reduce neutrophil activity and chemotaxis by interfering with lysosomal activity, inhibit neutrophil enrichment in inflammatory areas, and reduce phagocytosis, thus achieving anti-inflammatory effect (Nonaka et al. 2014). Colchicine is only recommended for the treatment of acute GA episodes and is often ineffective for other common inflammation and GA (Wang et al. 2014). Because the toxic dose and therapeutic dose are not different, it is often recommended to be used in low doses. Large doses of colchicine may cause gastrointestinal adverse reactions, rhabdomyolysis, liver, kidney, bone marrow function damage, and other side effects; therefore, the person of the disease such as dysfunction of liver and kidney should be applied carefully (Wang et al. 2017). Corticosteroids are recommended for short-term use, especially in patients with coexisting medical conditions who cannot use the first two drugs, in order to control inflammation while avoiding complications as much as possible. NSAIDs can also be used in

combination with intra-articular corticosteroids, oral steroids, or colchicine to better inhibit the acute onset of GA (Yamaguchi et al. 2022). In recent years, IL-1 blocking drugs have played a good anti-inflammatory effect in several clinical trials (Richette et al. 2011; Zhang et al. 2021), but their clinical application is limited due to a variety of reasons, such as high cost, too short half-life, low bioavailability, infection caused by multiple injections, and poor patient compliance (Wang et al. 2021).

2.4.2 Progress and Limitations of Exercise Therapy in GA

For patients with GA, regular exercise is essential, although pain and functional limitations can make movement more difficult. Exercise can reduce pain, maintain muscle strength and endurance around the joint, help to reduce pain, reduce stiffness of the joint, prevent functional decline, reduce the incidence of cardiovascular and cerebrovascular events, and improve mental state and quality of life.

In the acute phase of GA, patients should be guided to rest reasonably and perform isometric contraction of the muscles around the joint. Avoid weight-bearing activities in the affected joints and properly perform isometric muscle contraction training to maintain muscle state. Take the knee joint as an example, it is advisable to rest in the acute phase and try to avoid long-term standing, walking, and other knee weight-bearing activities, and do straight leg hooking and foot training to maintain the condition of muscles around the knee joint. In the non-acute phase of GA, the patients were guided to carry out exercise and joint function rehabilitation training. Patients with GA should follow the FITT principle. F (frequency): aerobic exercise 3–5 times/week, resistance training 2–3 times/week, flexibility training daily. I (Intensity): mild-to-moderate intensity aerobic exercise and low-intensity resistance training. Exercise testing is recommended for those over 45 years of age with multiple cardiovascular and cerebrovascular risk factors. T (time): ≥ 150 min per week. T (type): aerobic exercise should be emphasized. Studies have shown that regular exercise can reduce uric acid levels and prevent gout attacks (Kakutani-Hatayama et al. 2017) (Fig. 2.4).

2.5 Conclusion

Articular cartilage provides frictionless movement between articular surfaces during joint movement and acts as a cushion for the subchondral bone in the weight-bearing areas of the body, so maintaining healthy cartilage is important. Cartilage tissue lacks vascular, lymphatic, and nerve distribution, so it is difficult to repair naturally once damaged. The various treatments described in this paper, although each has its own advantages, are not completely effective in repairing cartilage tissue. In terms of function, the repaired tissue cannot meet the mechanical requirements of articular cartilage, and the integration with the original tissue is poor, and the difference between the old and new tissues is obvious. It can be predicted that these new tissues may be damaged again during the use of the joint. In cell transplantation therapy, cell

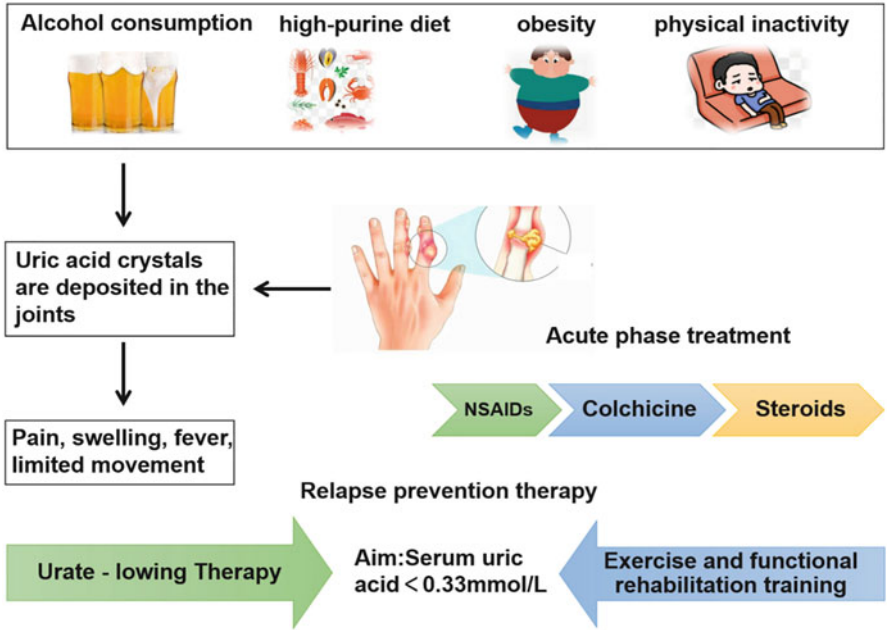


Fig. 2.4 Etiology, pathology, clinical manifestations, and treatment of gouty arthritis (GA). The precipitating factor of GA includes alcohol, a high-fat diet, obesity, and lack of exercise. The pathology of GA is the deposition of monosodium urate crystals in the joints. GA presents with severe joint pain, accompanied by signs of redness, swelling, fever, and dysfunction. The treatment of GA includes urate-lowering therapy, non-steroid anti-inflammatory drugs, colchicine, corticosteroid, and functional rehabilitation training

adhesion scaffold material is a difficult problem, the choice of support material may determine the success of transplantation, they must have a biocompatible, biodegradable and certain mechanical strength, porous load cells, and cell adhesion of good features, but is very not easy to find if it meets the requirements of these materials and finally the fixation of the graft at the site of injury. Many experiments have reported that osteophytes grow around the joint after the transplantation of cells or cell/scaffold complexes. It is very likely that the graft is not fixed at the defect site, but falls onto the articular surface and forms bone tissue under the stimulation of the surrounding environment. However, with the development of biology, medicine, materials engineering, and other disciplines, these problems of articular cartilage defect repair will be solved.

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Strategies to Control Mesenchymal Stem Cell Differentiation for Regenerating Phenotypically Defined Articular Cartilage

3

Zheng Yang, Deepak Raghothaman, Daryl Jimian Lin,
and Eng Hin Lee

Abstract

Articular cartilage has limited intrinsic ability for self-regeneration, and treatments to restore fully functional cartilage remain a challenge. Mesenchymal stem cells (MSCs), given their relative ease of derivation, proliferative capacity, and differentiation potential, offer a promising alternative therapeutic approach for cartilage repair. Despite numerous efforts evaluating various factors such as culture conditions, differentiation cocktails, and properties of three-dimensional (3D) substrates, MSC-based approaches for cartilage repair have yet to achieve the consistency and effectiveness for widespread clinical use. MSC-derived cartilage tissue does not resemble physiological cartilage, typically yielding mixed hyaline/fibrocartilaginous phenotypes, with inherent tendency toward hypertrophy. The regenerated neocartilage suffers from sub-optimal biochemical content and mechanical strength, in comparison with chondrocyte-derived tissue. Further, the lack of a hierarchical architecture with zonal cellular and extracellular matrix composition and properties in the engineered tissue limits the restoration of normal tissue function. This chapter provides an overview of key approaches for manipulating chondrogenic differentiation of MSCs, with emphasis on recent progress in (1) the enhancement of extracellular matrix (ECM) and mechanical

Z. Yang · D. J. Lin · E. H. Lee (✉)

Tissue Engineering Program, Life Sciences Institute, National University of Singapore, Singapore, Singapore

Department of Orthopaedic Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

e-mail: leeh@nus.edu.sg

D. Raghothaman

Department of Orthopaedic Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

strength of neocartilage tissue; (2) the prevention of cartilage hypertrophy; and (3) the generation of phenotypically distinct cartilage. Critical insights on refining regenerative approaches for articular cartilage repair, such as the requisite composition and amenability of 3D matrices for dynamic cellular and morphogenetic events, are addressed.

Keywords

Cartilage tissue engineering · Mesenchymal stem cells · Chondrogenesis · Biomimetic materials · Cartilage hypertrophy · Zonal phenotype

3.1 Introduction

Articular cartilage is an avascular connective tissue, with limited intrinsic ability for self-repair or regeneration. Chondral lesions that do not penetrate the underlying subchondral bone are unable to heal spontaneously as progenitor cells are not able to migrate to the injured site from the underlying bone marrow. Even full-thickness defects undergo only transient healing and are often replaced with fibrous tissues or, at best, result in fibrocartilage regeneration (Hunziker 2002; Ahmed and Hincke 2010). Fibrocartilage differs significantly in its biochemical composition from normal hyaline cartilage, exhibiting inferior mechanical properties. Focal lesions can thus progressively lead to debilitating osteoarthritis.

A critical consideration of native cartilage tissue and its organization is pivotal for devising optimal strategies for functional tissue repair. Cartilage tissue is comprised of a single cell type, chondrocytes, which are embedded in a matrix composed of abundant collagen and proteoglycans (PGs). Despite its simple appearance, articular cartilage exhibits significant heterogeneity, comprising of superficial, middle (transitional), and deep zones overlaying the calcified layer (Fig. 3.1). The density, morphology, and metabolic activity of the cells, as well as the composition and structural arrangement of the extracellular matrix (ECM) components, vary greatly between these zones (Hayes et al. 2007; Klein et al. 2009). The superficial zone (constituting the top 10–15% of total cartilage thickness) contains flattened chondrocytes with collagen fibrils aligned parallel to the articulating surface. Chondrocytes in the superficial zone produce superficial zone protein [SZP, also known as proteoglycan 4 (PRG-4) and lubricin] which acts as a lubricant for efficient gliding motion during joint movement (Greene et al. 2011). The middle zone (40–50% of total cartilage thickness) contains more rounded chondrocytes, and thicker collagen fibrils with random orientation. The deep zone (30–40% of total cartilage thickness) is made up of large, spherical chondrocytes in columnar arrangement, embedded in a dense ECM rich in PGs, with thick collagen fibrils aligned perpendicularly to the articulating surface. While PG concentration increases along the cartilage depth, collagen content (per wet weight) does not change significantly but displays depth-dependent increases in hydroxylysine and hydroxylysyl pyridinoline cross-links (Bank et al. 1998). The presence of other minor collagens

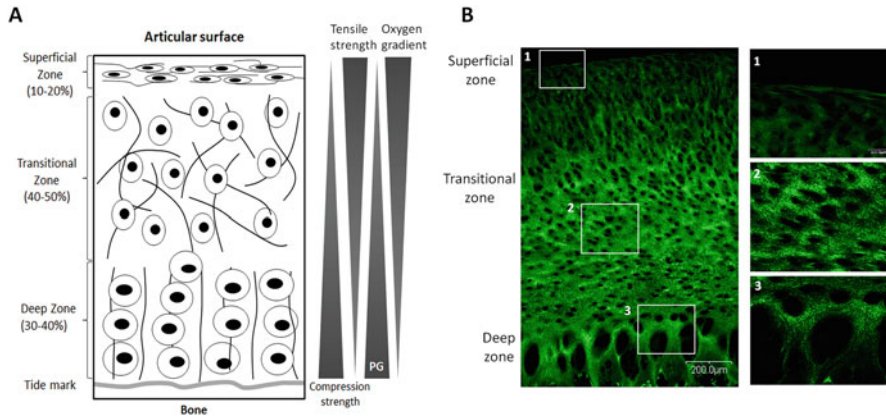


Fig. 3.1 (a) Schematic diagram showing zonal differences in mature articular cartilage. Variations in chondrocyte morphology, collagen fiber arrangement, proteoglycan content, oxygen gradient, tensile, and compressive strength are illustrated. PG; proteoglycans. (b) Tissue section of porcine native articular cartilage, where the zonal differences in collagen type II organization are demonstrated by SHG imaging (scale bar = 200 μ m). Regions 1–3 are five times optical magnifications, with region 1 showing parallel organization of Col II fibrils in superficial zone. Regions 2 and 3 show middle, mid-to-deep zones with random organization, with region 4 showing perpendicular organization of Col II fibrils from the deep zone

isoforms, such as type IX and XI, and *cartilage* oligomeric matrix protein (COMP), plays critical roles in the regulation of fibril size, inter-fibril cross-linking, and interactions with PGs (Blumbach et al. 2009; Haleem-Smith et al. 2012), conferring the characteristic compressive strength and dimensional stability of the articular cartilage tissue (Opolka et al. 2007; Amanatullah et al. 2012). In mature articular cartilage, matrix stiffness ranges from 80 kPa in the superficial zone to 2.1 MPa in the middle zone and 320 MPa in the calcified zone (Schinagl et al. 1997). Furthermore, oxygen tension falls steeply with distance from the cartilage surface, which is dependent on the balance between the rate of oxygen transport through cartilage and the rate of consumption by cells (Zhou et al. 2004; Malda et al. 2003).

Cell-based approaches to regenerate cartilage tissue for treating chondral defects offer an alternative solution for cartilage repair. However, despite efforts by researchers over the last three decades, achieving fully functional cartilage tissue repair remains a significant challenge. Approaches involving culture-expanded, autologous articular chondrocytes suffer from limited availability, associated donor site morbidity, loss of phenotypic maintenance during expansion, and low yields. Such shortcomings present major challenges to viable cartilage repair, especially for critical-size defects (Andrades et al. 2012; Darling and Athanasiou 2005). Mesenchymal stem cells (MSC), given their relative ease of derivation from various sources, proliferative capacity, and differentiation potential, offer a promising alternative for cartilage repair (Johnstone et al. 1998; Sekiya et al. 2002; Wakitani et al. 1994; Hui et al. 2004; Nejadnik et al. 2010). However, application of solely MSC-based approach has yet to facilitate cartilage repair that restores native

phenotype and function, but has resulted in neocartilage tissue with sub-optimal biochemical content and mechanical strength, when compared to chondrocyte-derived cartilage (Huang et al. 2009, 2010a; b; Chiang et al. 2011). Implantation of MSCs in animal models has often resulted in predominantly non-hyaline cartilaginous repair at the cartilage defect site (Steck et al. 2009; Zscharnack et al. 2010). The lack of robust, hyaline characteristics in stem cell-derived neocartilage stems from (a) the predisposition of MSC to form mixed hyaline/fibrocartilaginous phenotypes, with the co-expression of collagen type II (Col II) and collagen type I (Col I) (Vandenabeele et al. 2003; Steck et al. 2005); and (b) the innate propensity of MSC-derived chondrocytes to undergo terminal hypertrophic development, switching from generating cartilaginous matrix to a osseous matrix rich in collagen type X (Col X), ALP, and calcification (De Bari et al. 2004; Pelttari et al. 2006; Dickhut et al. 2009; Goldring et al. 2006).

Further, stem cell-derived cartilage tissue has been found to be comprised of a homogenous matrix, with bulk properties that have little resemblance to the native articular cartilage (Klein et al. 2009), lacking the hierarchical, depth-dependent variations in cellular phenotype, ECM composition, and collagen fiber orientation. The differences in the specific behavior of chondrocytes derived from various depths or zones of articular cartilage have been well established (Aydelotte and Kuettner 1988; Darling et al. 2004). The impetus on regeneration of zonally defined cartilage has been demonstrated by *in vitro* studies performed with chondrocyte subpopulations derived from different zones of cartilage (Kim et al. 2003; Sharma et al. 2007; Ng et al. 2009). Stratified hydrogels incorporating superficial and deep zone chondrocytes demonstrated better shear and compressive properties than homogeneous constructs. Further, augmentative signaling between zonal populations has been reported to aid the maintenance of typical tissue traits and function (Kim et al. 2003; Sharma et al. 2007; Ng et al. 2009; Blewis et al. 2007). These studies suggest that replicating the zonal hierarchy of native articular cartilage may improve functional properties of engineered cartilage (Klein et al. 2009; Schuurman et al. 2015). Thus, for stem cell-based cartilage regeneration, the ability to control and direct MSC differentiation into phenotypically defined cartilage will be paramount for the enhancement of the mechanical properties and the overall function of the regenerated cartilage.

Although a wide variety of culture conditions, differentiation factors, scaffolds, and bioreactors have been studied to promote cartilage regeneration, effective and reliable differentiation strategies to yield tissues emulating native cartilage properties remain to be developed. In considering the complex biological process of stem cell differentiation to yield zone-specific cartilaginous tissue, current research is showing that rather than a single or combination of a few growth factors/signaling pathways, cascades of biochemical interactions involving extensive, overlapping regulatory networks and their cross-talk and temporal activation are responsible for driving and regulating differentiation. Optimization of growth factor cocktails, or the activation of appropriate signaling pathways is time-consuming and may not be cost-efficient and may require additional factors to achieve the desired control over cartilage differentiation. Instead, manipulation of cell culture conditions that incorporate

developmental and/or physiological factors may provide a more distributed and pragmatic approach to regulate cellular differentiation. It also presents the possibility of triggering cascades of molecular responses, rather than local networks based on single gene or protein, to control MSC chondrogenesis. This review will summarize the different approaches to manipulate the chondrogenic differentiation of MSCs, with emphasis on recent progress in (1) the enhancement of ECM and mechanical strength of neocartilage tissue; (2) the prevention of cartilage hypertrophy; and (3) the generation of phenotypically distinct cartilage.

3.2 Spatial and Temporal Influence of Bioactive Factors

Biochemical regulatory factors, either as supplements to culture medium or incorporated within scaffolds to deliver the stimulating cues, play an important role in regulating *in vitro* chondrogenesis. Candidate bioactive factors, which stimulate chondrogenic differentiation and augment cartilage matrix synthesis, include members of the transforming growth factor (TGF)- β superfamily, bone morphogenetic proteins (BMPs), and growth differentiation factors (GDFs), as well as insulin-like growth factors (IGFs), Wnt proteins, and fibroblast growth factors (FGFs) (Goldring et al. 2006; Steinert et al. 2008; Bobick et al. 2009). Growth factor-induced chondrogenesis of MSC has been demonstrated to innately undergo endochondral ossification, often resulting in undesirable hypertrophy and ossification (De Bari et al. 2004; Pelttari et al. 2006; Dickhut et al. 2009; Bian et al. 2011a). Thus, an optimal tissue engineering strategy would entail the achievement of synergistic effects between site-specific application, and tight regulation of temporal exposure to specific growth factors, for the enhancement of chondrogenic differentiation and maintenance of their hyaline, functional phenotype.

The effect of various members of BMP and TGF family has been shown to induce the formation of cartilaginous tissue with different cellular phenotypes and gene expression profiles (Shintani and Hunziker 2007; Schmitt et al. 2012). TGF- β 1 was shown to enhance the BMP-2-induced chondrogenesis, improving the hyaline-like properties of the neocartilage, and arresting subsequent hypertrophic differentiation of MSCs (Schmitt et al. 2012). TGF- β -induced MSC chondrogenesis is not immune to hypertrophy development (Pelttari et al. 2006; Bian et al. 2011a). Temporal co-activation of the TGF- β signaling pathway with β -catenin controlled hypertrophic development, with prolonged co-activation of the two signaling pathways resulted in the suppression of hypertrophy, in comparison with transient co-activation, possibly through sustained synthesis of parathyroid hormone-related protein (PTHrP) and expression of cyclin D1 (Yang et al. 2012). The effect of PTHrP to suppress hypertrophy and calcification of cartilage was demonstrated with the co-delivery of PTHrP with TGF- β 3 (Bian et al. 2011b) and the temporal administration of PTHrP *in vivo* resulted in significant enhancement of cartilage repair with minimum hypertrophy, ossification, and matrix degradation (Zhang et al. 2013). In addition to promoting and regulating hyaline phenotype, growth factors could also influence the derivation of zonal cartilage phenotypes. TGF- β in combination with BMP-7 was

reported to upregulate the expression of superficial zone marker, PRG-4 (Andrades et al. 2012; Niikura and Reddi 2007; Khalafi et al. 2007). Concentration-dependent influence of growth factors, such as the use of a combination of high dose of TGF- β 1 (30 ng/mL) and IGF-1 (100 ng/mL), can lead to upregulation of the middle zone markers (Karimi et al. 2015). Thus, spatially controlled and localized delivery of defined factors at specific regions of the scaffold could be engineered to yield phenotypically distinct zonal cartilage.

3.3 Manipulation of Scaffold Microenvironment for Cartilage Tissue Engineering

Repair of critical-size defects typically involves delivery of cells in biodegradable, three-dimensional (3D) matrices, to physically contain the biologics within the repair site, and to serve as a temporary cartilage-like matrix structure that supports and promotes healing. Commercially available scaffolds have been specifically designed to be relatively simple in composition for early introduction into the clinical application, and in general catered for chondrocyte delivery. Given the different developmental status of MSC and terminally differentiated, mature chondrocytes, and the complexity of MSC chondrogenesis (Goldring et al. 2006; Bobick et al. 2009), scaffolds with specific properties that augment MSC-based cartilage regeneration would be required. Although a plethora of biomaterials have been fabricated and evaluated, in the form of hydrogels, fibrous, and macroporous scaffolds (Raghunath et al. 2007; Johnstone et al. 2013), few of them prove to promote sufficient functional cartilage regeneration. Increasing efforts have focused on developing functionalized scaffolds that incorporate instructive extracellular microenvironmental cues, for regulating stem cell morphogenesis.

3.4 Provision of Biomolecular Cues

One particular aspect of the microenvironment that heavily influences cell fate is the binding interactions that exist between cell receptors and components of the ECM. Numerous efforts involving the incorporation of various biomimetic components to mimic certain aspects of structure and function of native cartilage ECM and for the provision of suitable cellular microenvironment have been reported (Table 3.1). They were found to significantly influence cell–matrix and cell–cell interactions and dramatically alter the differentiation outcomes of MSCs. Inclusion of chondroitin sulfate (CS), a major glycosaminoglycan found in cartilage, resulted in an enhancement of cartilage-specific matrix production, with a significant down-regulation of chondrocyte hypertrophy (Wu et al. 2007; Varghese et al. 2008). Incorporation of CS or hyaluronic acid (HA) in alginate-based hydrogels resulted in the down-regulation of PRG-4 and Col I expression (Coates et al. 2012). Coating biomaterials with Col I or Col II (Wu et al. 2007; Noth et al. 2007; Bosnakovski et al. 2006; Hwang et al. 2011), or incorporating collagen mimetic peptide (Hwang et al.

Table 3.1 Effect of biomolecular cues on MSC chondrogenic differentiation

Biomolecular signals	Scaffold system	Elicited effect	References
Chondroitin sulfate	- Alginate microbead - PEG hydrogel	- Enhance MSC chondrogenic differentiation - Inhibit hypertrophy - Down-regulate PRG-4 and Col I	Wu et al. (2007), Varghese et al. (2008) and Coates et al. (2012)
Hyaluronic acid	- Collagen-based scaffold - Alginate - PEG	- Enhance MSC chondrogenic differentiation - Down-regulate PRG-4 and Col I - Inhibit ECM deposition	Matsiko et al. (2012), Nguyen et al. (2011a), Coates et al. (2012) and Hwang et al. (2011)
Collagen type II	- Alginate microbead - Col II hydrogel - PEG	Enhance MSC chondrogenic differentiation	Wu et al. (2007), Bosnakovski et al. (2006) and Hwang et al. (2011)
Collagen type I	- Col I hydrogel - Alginate/chitin IPC hydrogel	- Enhance MSC chondrogenic differentiation - Down-regulate PRG-4 and Col I - Enhance expression of COMP, Col IX	Noth et al. (2007), Raghothaman et al. (2014) and Raghothaman et al. (2014)
Collagen mimetic peptide	PEODA PEG	- Enhances MSC chondrogenic differentiation - Inhibits hypertrophy	Lee et al. (2008), Liu et al. (2010a) and Lee et al. (2008)
RGD peptide	Hyaluronic acid hydrogel	Low-density RGD enhances chondrogenesis	Kim et al. (2013)
Matrix metalloproteinase-sensitive peptides (MMP-pep)	PEG hydrogel	- Increase scaffold degradation - Improve collagenous matrices distribution - Increase dynamic compressive modulus	Nguyen et al. (2011a) and Bahney et al. (2011)
N-cadherin mimetic peptide	Hyaluronic acid hydrogel	- Promote early chondrogenesis of MSCs - Enhance cartilage-specific matrix production	Bian et al. (2011a)

2011; Lee et al. 2008), were shown to both enhance chondrogenic differentiation of MSCs and maintain the chondrogenic phenotype. Spatial incorporation of biochemical cues has profound effect on MSC chondrogenesis. Aligned incorporation of Col I in interfacial polyelectrolyte complexation (IPC)-based hydrogel fibers, by mediating early and uniform cell–cell interactions, elicited superior chondrogenesis and the generation of mature hyaline neocartilage, with notable down-regulation of fibrocartilaginous marker (Raghothaman et al. 2014).

Matrix-degradability and cell-induced remodeling can significantly affect cell morphology, spreading, and their migration (Buxton et al. 2007). A major limitation with many scaffolds is their resorption rates, which does not match the rate of matrix deposition by the encapsulated cells. Synthetic scaffolds are engineered to hydrolytically degrade at physiological pH, wherein bulk degradation often coincides with a precipitous drop in the construct mechanics. In order to provide for mechanical functionality and distribute forces during joint movement, in situ formed hydrogels for cartilage repair necessitate a high cross-linking density. High cross-linking density, however, generally yields smaller pore size within the scaffold, has been associated with reduced chondrogenesis, and inhibited matrix production and assembly, especially for collagen (Buxton et al. 2007; Erickson et al. 2009). Despite enhanced MSC chondrogenesis with the provision of biochemical cues, the reported deposition of ECM by polyethylene glycol (PEG) or HA hydrogel-encapsulated cells is restricted to the pericellular domain (Varghese et al. 2008; Hwang et al. 2011; Liu et al. 2010a; Nguyen et al. 2011a, b). The lack of well-distributed ECM can be overcome with an enzymatically degradable scaffold, by incorporating collagenase or matrix metalloproteinase (MMPs)-peptide substrates into scaffold design (Park et al. 2004; Lutolf et al. 2003; Patterson and Hubbell 2010; Sridhar et al. 2015). A bioresponsive hydrogel with cell-mediated degradation, aligned to the intrinsic cellular mechanisms in the chondrogenic differentiation of MSCs, was shown to induce more extensive collagenous deposition with increases in the dynamic compressive modulus of the engineered neocartilage construct (Sridhar et al. 2015; Bahney et al. 2011).

An alternate to laboratory-engineered 3D matrices with extracellular, biomolecular cues is the use of decellularized tissue explants, or in vitro cell-derived decellularized cartilage ECM, that contain tissue-specific ECM and growth factors niche (Xue et al. 2012) to modulate MSC behavior and differentiation (Cheng et al. 2014). MSCs cultured with cartilage matrix (Xue et al. 2012; Yang et al. 2008; Cheng et al. 2009), chondrocyte, or MSC-derived-decellularized matrix (Cheng et al. 2009; Lu et al. 2011) have resulted in substantial cartilaginous ECM deposition, comparable to pellet culture of MSCs. In addition, preconditioning stem cells on decellularized stem cell matrix were shown to enhance the in vitro and in vivo chondrogenic potential of expanded cells (Pei et al. 2013; Li et al. 2014). In theory, such approaches could also be extended to provide zone-specific decellularized matrices, for deriving phenotypically defined chondrocyte populations using MSC-based approaches (Huang et al. 2017).

3.5 Provision of Physical Cues

MSC chondrogenesis can also be controlled by manipulating the physical properties of the microenvironment. Structural variations include pore size, porosity of macroporous scaffold, fiber diameter, and orientation of fibrous scaffold, which affect the stiffness and surface topography. Such physical variations can influence cell attachment, cytoskeletal networks, and impart mechano-signaling that regulate the transcription of genes governing cell growth and differentiation (Discher et al. 2009; Guilak et al. 2009; Steward and Kelly 2015).

3.5.1 Substrate Elasticity

Stem cell lineage specification has been recognized to be directed by substrate elasticity (Engler et al. 2006). The effect of matrix stiffness on MSC chondrogenesis was described by Ghosh et al. (2009), employing nanofibrous silk protein scaffolds. MSCs reportedly sensed the subtle variations in morphology and stiffness of the silk matrices and were found to migrate and assume aggregated morphologies on more compliant matrices, mimicking early-stage chondrogenesis that resulted in heightened ECM production. Matrix elasticity was also reported to influence the chondrogenic phenotype of chondrocytes (Schuh et al. 2010). Development of HA- and PEG-based scaffolds with tunable mechanical and rheological properties has allowed the identification of an optimal, lower cross-linked matrix elasticity (Young's modulus at ~3–6 kPa) for MSC chondrogenesis (Toh et al. 2012; Bian et al. 2013a; Liu et al. 2021). Notably, MSCs and chondrocytes exhibit opposite trends in cartilage deposition in response to stiffness changes, with MSCs requiring soft hydrogels, while chondrocytes producing cartilage more effectively in a stiffer matrix of >20 kPa (Liu et al. 2021; Yang et al. 2017). A shift in MSC differentiation toward the fibrocartilage and fibrous tissue formation (Toh et al. 2012), or induction of calcification (Bian et al. 2013a), was reported with increasing cross-linking and matrix stiffness of HA hydrogels. Given that compressive modulus of full-thickness articular cartilage is depth-dependent (Schinagl et al. 1997), engineering scaffold material with a stiffness gradient that approximate the stiffness of different cartilage zones might allow the manipulation of MSC-derived neocartilage phenotypes.

3.5.2 Surface Topography

Topographic influence on cells to micrometer range features has been extensively studied and well established (Stevens and George 2005; Curtis and Wilkinson 1999). Advances in nanofabrication techniques have enabled the development of engineered surface topographies, with nanoscale features mimicking native ECM for controlling cell behavior (Nie and Kumacheva 2008; Norman and Desai 2006). Nanofibrous, electrospun polycaprolactone (PCL) scaffold with random orientation was found to support MSC chondrogenic differentiation (Li et al. 2005). Hyaline

cartilage matrix production was preferentially upregulated on randomly oriented nanofibers, whereas structurally anisotropic, aligned nanofibrous scaffold served as an instructive 3D pattern that promoted fibrochondrogenesis (Baker et al. 2010; Wise et al. 2009). Implantation of MSCs or chondrocytes on PCL nanofibrous scaffolds in a mini-pig model demonstrated that such nanofibrous scaffolds were well-tolerated in vivo, and fostered tissue regeneration (Li et al. 2009).

However, one of the limitations in employing electrospun fibers is their weak standardization of spatial topography. The ability to specifically and spatially control the patterning of nanoscale features is a prerequisite to allow identification of specific nanotopographies that regulate the formation of zonal cartilage phenotypes. Employing thermal nanoimprinting technology, Wu et al. showed that MSCs adopted distinct morphology and cytoskeletal structure on different nanotopographical patterns, with nanograting inducing formation of fibro/superficial zone-like cartilage, while nanopillar promoting cell aggregation and the formation of hyaline-like cartilage (Wu et al. 2014). Further, substratum stiffness of topographical pattern also contributes to influence the phenotypic development at the earlier stage of chondrogenic differentiation (Wu et al. 2017). Hyaline-like cartilage with middle/deep zone cartilage characteristics was generated on softer pillar substrate, while stiffer nanopillar material generated constituents of hyaline/fibro/hypertrophic cartilage. While fibro/superficial zone-like cartilage could be derived from nanograting of softer stiffness, stiffer nanograting resulted in insignificant chondrogenesis. These studies demonstrate the possibility of refining the phenotype of cartilage generated from MSCs by manipulating both the surface topography and material stiffness.

The nanotopographic substrate has been employed as a predifferentiation platform to generate phenotypically stable and zonally defined chondrogenic cells in vitro prior to implantation for cartilage regeneration. Wu et al. demonstrated that with an appropriate period of differentiation, two-dimensional nanotopographic patterns generated phenotypically stable chondrogenic cells, which, when implanted as stratified bi-layered hydrogel constructs in an osteochondral rabbit defect model, formed functionally superior cartilage tissue with clear zonal differences in the cell morphology and, in particular, distinct demarcation of collagen orientation (Wu et al. 2020). Such an approach demonstrates a relatively straightforward method of obtaining large quantities of zone-specific chondrocytes from MSCs to engineer a stratified cartilage construct that could recapitulate the zonal architecture of hyaline cartilage and can be applied to lesions of different size and shape.

3.6 Biomaterial Facilitation of Mesenchymal Condensation

One of the principal challenges in the field of cartilage tissue engineering is to recapitulate native developmental processes to establish stable cartilage phenotypes. Chief among such factors for MSC chondrogenesis is precartilage mesenchymal condensation, brought about by cell–cell interactions, involving neural cadherin (NCAD), and neural cell adhesion molecule (N-CAM) (Fig. 3.2a) (Goldring et al. 2006; DeLise et al. 2000). The widely adopted scaffold-free high-density micromass

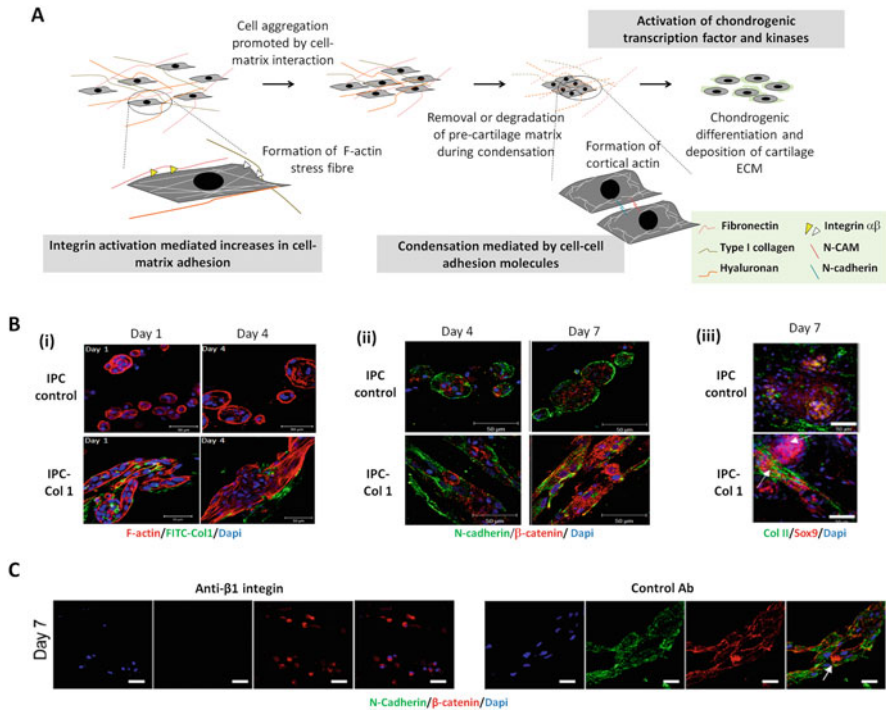


Fig. 3.2 (a) The process of chondrogenic initiation in stem cells. Aggregation of mesenchymal progenitors is mediated by cell–matrix interactions. Integrin activation mediated increases in cell–matrix adhesion, with associated fibroblastic morphology ensues cellular condensation. The initiation of condensation is marked by a shift in cell morphology, involving cytoskeletal rearrangement to form cortical actin. Concomitant removal or degradation of pre-existing mesenchymal ECM, and expression of NCAD and N-CAM molecules precede chondrogenic differentiation and ECM deposition. (b) Temporal changes in cell morphology of the encapsulated MSCs in IPC-Col I hydrogel mimicked the sequential changes in cell morphology and onset of chondrogenesis, as observed during in vivo chondrogenic process. (B-i) IPC hydrogel-mediated Col I presentation facilitated extensive and uniform cell alignment, and cell–matrix interactions, (B-ii) leading to precartilage condensations, (B-iii) thereby resulting in an early, robust onset of chondrogenic differentiation. (c) Integrin inhibition studies established the role of integrin-mediated cell–matrix interaction for MSC chondrogenesis in IPC-Col I hydrogels. Chondrogenic commitment was altered in IPC-Col I hydrogels treated with anti- β 1 integrin, as seen by the abrogation of N-cadherin expression, relative to treatment with control antibody (scale bar 50 μ m). [Adapted from Raghothaman et al. (2014)]

or pellet culture was developed to provide abundant cell–cell contacts, to emulate cellular condensations during chondrogenesis in vitro (Johnstone et al. 1998). By recapitulating such developmental process of mesenchymal condensation and the temporal fusion of mesenchymal bodies, production of centimeter-sized, anatomically shaped pieces of mechanically functional cartilage was reported by Bhumiratana et al. (2014). However, the inherent limitation with most 3D hydrogel

approaches for cartilage tissue engineering is the maintenance of spherical MSC morphology, with limited cell–cell interactions (DeLise et al. 2000; Tacchetti et al. 1992). In comparison with traditional pellet culture, the reduced cell–cell contacts, and activation of Notch and Wnt/ β -catenin pathways in PEG hydrogel, were found to correlate with its signaling and differentiation capacity (Chen et al. 2015). This perhaps accounts for the limited ECM production by the differentiated MSCs in PEG- or HA-based hydrogel studies (Varghese et al. 2008; Hwang et al. 2011; Liu et al. 2010a; Nguyen et al. 2011a, b).

Prior to condensation, aggregation of mesenchymal progenitors in hyaluronan- and Col I-rich ECM is mediated by cell–matrix interactions. Integrin activation increased in cell–matrix adhesion is a prerequisite for ensuing cellular condensation (Fig. 3.2a). The initiation of condensation is marked by a shift in cell morphology, involving cytoskeletal rearrangement to form cortical actin, with concomitant removal or degradation of pre-existing mesenchymal ECM, and expression of NCAD and N-CAM molecules (Goldring et al. 2006; DeLise et al. 2000; Tacchetti et al. 1992). Intracellular signaling initiates the transition from chondroprogenitor cells to a fully committed chondrocytic phenotype, by the activation of chondrogenic transcription factors and certain kinases to initiate the deposition of cartilaginous ECM (Goldring et al. 2006; Bhattacharjee et al. 2015). The importance of cell–matrix and cell–cell interaction in promoting robust MSC chondrogenic differentiation has been recognized in the study involving IPC-Col I hydrogel (Raghothaman et al. 2014). Temporal changes in cell morphology of the encapsulated MSCs in IPC-Col I hydrogel mimicked the sequential changes, recapitulating developmental events in *in vivo* chondrogenic process (Fig. 3.2a), including the enhanced cell–cell interactions and the onset of N-cadherin/ β -catenin-mediated chondrogenic induction and differentiation (Fig. 3.2b, c), resulting in superior chondrogenesis and the generation of mature hyaline neocartilage (Raghothaman et al. 2014). The importance of N-cadherin-mediated cell–cell interaction in MSC-based cartilage tissue development was also demonstrated by the functionalization of a hyaluronic acid hydrogel with conjugation of N-cadherin mimetic peptides (Table 3.1), which promoted both early MSC chondrogenesis and cartilage-specific matrix production within the hydrogel construct (Bian et al. 2013b).

Biomaterial-induced cellular aggregation can be achieved through manipulation of the physical environment of the cells. Precedents include the nanopillar surface topography (Wu et al. 2014), mechanically compliant flexible electrospun matrix (Ghosh et al. 2009), weakly cross-linked collagen-based hydrogels (Zhang et al. 2012), or the oriented ligand provided by IPC hydrogel (Raghothaman et al. 2014), all of which facilitated cellular migration and aggregation, ubiquitously resulting in the enhancement of MSC chondrogenic differentiation. It is thus essential for future 3D scaffold designs to integrate features that facilitate the critical condensation process for robust stem cell chondrogenesis, and aid the regeneration of a mechanically functional neocartilage tissue.

3.7 3D Composite Multilayered Scaffolds

Numerous studies have devised 3D composite multilayered scaffold with variation in zone-specific growth factors, matrix stiffness, fiber, and structural orientation, to direct MSC differentiation into zonally organized cartilage. PEG-based hydrogel systems have been employed to demonstrate the potential of spatially incorporating specific combinations of natural and synthetic biomolecular matrix, including degradative cues, in a layered scaffold microenvironment (Nguyen et al. 2011a, b). Such strategies aided in directing MSC differentiation into multiphasic phenotypes of chondrocytes, resembling cells with characteristic properties from the superficial, transitional, and deep zones of articular cartilage. Employing sequential electrospinning technology, microstructural trilaminar scaffolds were fabricated with depth-dependent variations in orientation and fiber sizes in a continuous construct, which resulted in the formation of neotissue mimicking some organizational characteristics of native cartilage (McCullen et al. 2012). Similarly, a bi-layered PCL scaffold with overlaid, aligned microfiber layers enhanced the mechanical and surface properties of the macroporous scaffold (Steele et al. 2014). Zonal analysis of these scaffolds yielded region-specific variations in chondrocyte number, glycosaminoglycan (GAG)-rich ECM, and chondrocytic gene expression, demonstrating the potential of multiphasic structural organization for the regeneration of a hierarchically organized articular cartilage tissue.

A common limitation associated with both multilayer hydrogels and scaffolds, however, is that the nanoporous hydrogel/scaffold network physically constrains cell–cell interaction among the encapsulated cells, delaying the new matrix deposition, and limiting it only to pericellular regions even after weeks of *in vitro* culture (Sharma et al. 2007; Ng et al. 2009; Nguyen et al. 2011b). This limitation results in relatively low compressive moduli (~10–90 kPa) of resulting cartilage, which remain one order of magnitude lower than native cartilage (Hoenig et al. 2013). Toward this end, a macroporous, elastomeric microribbon (μ RB)-based scaffold was constructed that has shock absorbance properties, and support MSC-based cartilage formation with much faster increase in mechanical strength compared to conventional hydrogels (Conrad et al. 2018). A multicompositional, spatially patterned μ RB scaffold of mixed ratios of chondroitin sulfate (CS) and gelatin (GEL) μ RBs was further demonstrated to regenerate cartilage with biochemical, mechanical, and morphological zonal organization (Gegg and Yang 2020).

3.8 Manipulation of Cell Culture Conditions

3.8.1 Co-Culture Platform

A major challenge with MSC-based strategies for cartilage tissue engineering is the production of transient cartilage, which readily progresses to hypertrophy and calcification, reminiscent of endochondral ossification (Pelttari et al. 2006; Dickhut et al. 2009). Co-culture of MSCs with chondrocytes has been considered as a

promising strategy to mitigate such hypertrophic development (Hubka et al. 2014). The cellular interaction between the cell types regulates their chondrogenic phenotype, resulting in robust cartilage formation, while also lowering the number of mature chondrocytes required. Co-culture of low amounts of chondrocytes (20%) with adult stem cells was found to result in robust MSC chondrogenesis while restraining hypertrophy in MSC-derived neotissue (Bian et al. 2011b; Acharya et al. 2012; Fischer et al. 2010). PTHrP secreted by chondrocytes was identified as the key factor for the observed anti-hypertrophic effects (Fischer et al. 2010). With heightened sensitivity to chondrogenic stimuli, results also point to mutual benefits for phenotype maintenance in both cell types (Dahlin et al. 2014a). While chondrocytes inhibit hypertrophy of differentiating MSCs and enhance MSC chondrogenesis (Liu et al. 2010b; Meretoja et al. 2012), the presence of MSCs in co-cultures exerts a trophic effect that stimulates chondrocyte proliferation (Acharya et al. 2012; Wu et al. 2011, 2012). Taking advantage of the metabolically more active MSCs, MSCs in a co-culture system can also exert paracrine effects for remodeling the surrounding matrix. Chondrocytes co-encapsulated with a lower number of MSCs in a PEG-norbornene hydrogel engineered with MMP-degradable peptide were shown to have enhanced local degradation of hydrogel, compared to hydrogel with chondrocytes alone, resulting in widely spread and interconnected ECM formation (Sridhar et al. 2015). Co-implantation of MSCs and a low number of chondrocytes in osteochondral defects *in vivo* led to hyaline-like cartilage repair, in comparison with implantation of MSCs alone (Dahlin et al. 2014b; Rogan et al. 2020). By achieving the quality of cartilage repair comparable with implantation of chondrocytes alone, these studies demonstrated the potential of articular chondrocyte-MSC co-implantation for the repair of cartilage defects.

3.8.2 Oxygen Tension

While tissue engineering approaches have mostly involved expansion and differentiation of MSCs under normoxic conditions (20% O₂), the *in vivo* cartilage niche is hypoxic (1–6% O₂). Hypoxia has generally been shown to promote chondrogenesis of MSCs and increase cartilaginous matrix synthesis (Kanichai et al. 2008; Robins et al. 2005; Hirao et al. 2006). Low oxygen tension has also been shown to suppress hypertrophy of chondrogenically induced MSCs (Hirao et al. 2006; Gawlitta et al. 2012; Sheehy et al. 2012). Using an explanted fetal tibiae culture, Leijten et al. demonstrated that hypoxia increased the amount of the resting zone chondrocytes, mRNA levels of hyaline cartilage-related genes, and the size of the cartilaginous epiphysis, while reducing the hypertrophic zone (Leijten et al. 2012). Activation of p38 MAPK pathway (Hirao et al. 2006) or SOX9 activity via hypoxia-inducible factor 1 alpha (HIF-1 α) (Kanichai et al. 2008; Robins et al. 2005; Duval et al. 2012) was reported to result in the enhanced chondrogenesis, while hypoxia was shown to inhibit hypertrophy by activating HDAC4 and Nkx3.2, suppressing SMAD signaling pathways (Hirao et al. 2006; Kawato et al. 2011), or via stimulation of the PTHrP–MEF2C pathway (Browe et al. 2019). Gene network analyses revealed that

oxygen tension resulted in metabolic programming of the MSCs, directing their chondrogenic differentiation into articular- or epiphyseal cartilage-like phenotypes (Leijten et al. 2014). The distinct cartilage phenotypes were preserved upon implantation in mice, indicating that metabolic programming of MSCs by oxygen tension could provide a simple yet effective mechanism to direct the chondrogenic differentiation to either transient hypertrophic cartilage, or stable articular-like cartilage phenotype. Notably, the effect of hypoxia on hypertrophy could also be influenced by the biochemical composition of the microenvironment, such as the concentration of HA hydrogels (Zhu et al. 2014). Hypertrophic differentiation and neocartilage calcification were inhibited in hydrogels with lower HA concentration, while substantially enhanced MSC hypertrophy, leading to elevated tissue calcification, was observed in hydrogels with higher HA concentrations. This study underscores the requirement for a thorough evaluation on the effects of oxygen tension, in the context of specific scaffold microenvironments employed.

To date, there is little direct evidence on the effect of oxygen tension in regulating the zonal phenotype of chondrogenically induced MSCs. Evidence from studies using cartilage-derived chondrocytes has, however, shown that zonal chondrocytes retain their phenotypic differences during *in vitro* cultivation more effectively under hypoxic culture (Schrobbach et al. 2012). Low oxygen tension was shown to reduce the gene expression of superficial zone protein, PRG-4, in chondrocytes (Hatta et al. 2014; Mhanna et al. 2013), indicating that physiological distribution of PRG-4 in the articular surface of cartilage may be due to the oxygenated environment of superficial zone cartilage. Modulating both the oxygen tension (via spatial confinement) and mechanical environment through the depth of MSC-seeded hydrogels, Thorpe et al. demonstrated an increase in PRG-4 deposition toward the top surface of oxygenated tissues, higher GAG accumulation in the bottom of constructs, with suppression of hypertrophy and calcification throughout the construct (Thorpe et al. 2013). Given the fall in oxygen levels in native cartilage, from 7 to 10% at the superficial layer, to near 0.1% adjacent to the subchondral bone (Zhou et al. 2004; Malda et al. 2003), manipulating local oxygen environment *in vitro* might present an alternate strategy to influence the pattern of cartilage phenotype acquired during MSC differentiation. It might also aid the formation of a zonally stratified neotissue.

3.8.3 Dynamic Stimulation

Under physiological conditions, cartilage is subjected to a range of mechanical loading such as hydrostatic pressure, compressive, and shear forces, causing cell and tissue deformation and changes in fluid flow. Cartilage regeneration strategies must therefore consider the biomechanical environment and physical stimuli. A variety of bioreactor systems have been designed for the perfusion of nutrients and metabolites, and to exert mechanical and hydrodynamic forces, to study their influence on stem cell chondrogenesis (Schulz and Bader 2007). Mechanical stimulation including cyclic compression (Huang et al. 2005; Mouw et al. 2007; Pelaez et al. 2009; Li et al. 2010a) and hydrostatic pressure (Angele et al. 2003; Miyamishi

et al. 2006; Sakao et al. 2008) was found to promote MSC chondrogenesis, by acting partially through the TGF- β pathway (Huang et al. 2005; Li et al. 2010a). However, unlike chondrocytes, compression applied before the deposition of sufficient pericellular matrix by chondro-differentiating MSCs was reported to have an inhibitory effect on MSC chondrogenesis (Haugh et al. 2011; Huang et al. 2010c). Delayed dynamic loading provided after a preculture period of chondrogenic induction was shown to be beneficial for MSC chondrogenesis (Huang et al. 2010c; Li et al. 2012). The reported increase in tissue mechanical strength was likely to be contributed by higher Col II and proteoglycan production, and enhanced collagen cross-linking mediated by increases in Col IX, Col XI, and pyridinoline (Yan et al. 2009). However, contrasting effects of cyclic compressional stimulation on hypertrophic development of MSC have been reported. Long-term dynamic loading was shown to induce hypertrophy development (Haugh et al. 2011), while other studies reported the stabilization of chondrogenic phenotype by dynamic compression (Bian et al. 2012; Zhang et al. 2015) and hydrostatic pressure (Vinardell et al. 2012). A study with elastomeric polyester scaffolds suggested that compression-driven hypertrophic development involves cross-talk between TGF- β /SMAD2/3 signaling and integrin-ECM interactions, regulating the suppression of the BMP/GDP and integrin/FAK/ERK signaling (Zhang et al. 2015). Such inhibition of hypertrophic development by mechanical compression correlated with the outcomes of an *in vivo* study involving orthotopic transplantation of MSC, wherein Col X-positive chondrocytes were found only at the osteochondral interface (Steck et al. 2009). The reported variability in outcomes point to (1) the sensitivity of MSCs to dynamic parameters, such as loading intensity, duration, and frequency; (2) differences owing to the properties of the scaffolds employed; and (3) the differentiation status of MSCs subject to the mechanical stimuli.

Physiological mechanic loading has been described as a pivotal factor for the development of zonally defined cartilage in mature animals. Cartilage tissue forms during embryonic chondrogenesis neither consist of multiple zones, nor the unique Benninghoff collagen architecture associated with articular cartilage. Such features are absent at birth, but develop during skeletal maturation in postnatal development (Rieppo et al. 2009; Julkunen et al. 2010), shaped by articular motion exerting a combination of compressive, tensile, and shear loading. Thus, such factors can also significantly influence the chondrogenic differentiation of MSCs during articular cartilage repair. Apart from compression loading, mechanical shear deformation, long-term intermittent shear deformation (Nugent et al. 2006; Grad et al. 2006), and fluid-induced shear stress (Ogawa et al. 2014; Gemmiti and Guldberg 2009) were shown to improve the quality of cartilaginous tissue formed *in vitro* (Waldman et al. 2003; Fitzgerald et al. 2006, 2008), and specifically inducing the expression of superficial zone-specific PRG-4.

The combined interplay between load and articular oscillation shearing, and their relationship to chondrocyte responses was explored with the employment of multi-axial bioreactor, that superimposed surface shearing on cyclic axial compression, mimicking *in vivo* articulation (Grad et al. 2006). Upregulated mRNA expression of cartilage oligomeric matrix protein (COMP) and PRG-4 was observed in such

studies, which were not detected when only compressional loading was provided. The application of shear superimposed upon dynamic compression led to significant increases in MSC chondrogenic gene expression (Li et al. 2010a; Schätti et al. 2011), with the phenotype of the derived neocartilage tissue dependent on the frequency and amplitude of the compression and shear stress (Li et al. 2010b). Despite the numerous reports of dynamic stimulation on MSC chondrogenesis, the precise and systemic mechanisms for such mechano-transduction remain largely unclear, especially involving the multiaxial stimulation. Eventual adaptation of dynamic stimulation to improve cartilage tissue engineering outcomes will require systematic studies on the stimulation parameters to be adopted, with context to the specific scaffold microenvironment employed. Such studies could also elucidate the underlying mechanisms at play, connecting the external dynamic stimulation to the intracellular molecular signaling networks, which aid the derivation of zone-specific chondrogenic phenotypes.

3.9 Multifactorial Approach in Stimulating MSC Chondrogenesis

Research efforts have moved beyond investigating the effect of single factors, such as scaffold biochemical and biophysical properties, various culture conditions, and dynamic stimulation on MSC chondrogenesis, to evaluate the influence of combinatorial factors on chondrogenic outcomes. Such studies include investigations on the effect of hypoxia and biochemical microenvironment on MSC chondrogenesis and hypertrophy (Zhu et al. 2014), modulation of both oxygen tension and mechanical environment (Thorpe et al. 2013), the interplay between nanotopographical microenvironment and flow stimulus (Zhong et al. 2013), and interaction of various biophysical cues including substrate stiffness, topography, dimension, and biochemical cues (Wu et al. 2017; Gegg and Yang 2020; Wang et al. 2014; Moeinzadeh et al. 2016; Li et al. 2013). The importance for a thorough evaluation on the effects of particular culture condition, in the context of specific scaffold microenvironments, was aptly demonstrated by Zhu et al. in which the effect of hypoxia on MSC chondrogenesis and hypertrophic development were dependent on the concentration of HA hydrogels employed (Zhu et al. 2014).

In the context of multifactorial studies, physical and biochemical cues of scaffolds may interact in a non-linear manner to influence MSC chondrogenesis (Wang et al. 2014; Nii et al. 2013). Using hydrogel platforms with independently tunable mechanical stiffness and biochemical composition (Tong and Yang 2014), it was demonstrated that increasing HA concentrations on softer hydrogels resulted in substantial upregulation of aggrecan and Col II expression, in a dose-dependent manner. This trend was, however, reversed in HA-containing stiffer hydrogels (Wang et al. 2014). These studies highlight the importance of creating platforms with independently tunable microenvironmental cues, to delineate the influence of one factor relative to the other, in bringing about cellular responses. Such approaches could decipher the influence of different microenvironmental cues, and their

combinations, on the regulation of stem cell fate in 3D, and aid the identification of optimal niche cues that promote desirable cellular processes and tissue regeneration.

3.10 Future and Conclusion

It has been recognized that functional cartilage regeneration with long-term durability requires the recapitulation of physiological developmental process, and recreating the heterogeneity of native tissue in its cellular phenotypes and distribution, anisotropic ECM composition, and mechanical properties (Fig. 3.3). Although a wide variety of differentiation factors, scaffold platforms, bioreactors, and culture conditions has been studied to promote cartilage synthesis, effective and reliable strategies yielding tissues with properties matching native tissue remain to be developed. Noteworthy is the increasing emphasis on the provision of optimal biomaterial composition and compliant 3D microenvironments to better facilitate chondrogenic phenotypes and improve the function of engineered constructs. Combinatorial studies of multiple cues, including physical, biochemical, and physiological factors on stem cell responses, have been reported to augment MSC chondrogenesis and to elucidate the link between these factors and regulatory networks. As yet, the impact of such biomechanical and biochemical regulators on

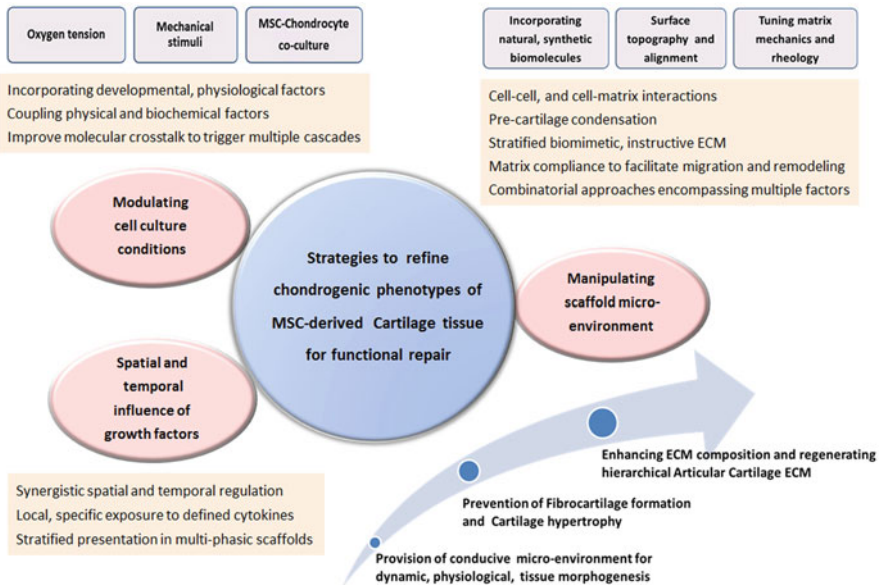


Fig. 3.3 An overview of experimental strategies to refine MSC-derived chondrogenic phenotypes for functional cartilage repair. Strategies can fall into three main categories, namely, modulating cell culture conditions to incorporate developmental factors, regulating spatio-temporal exposure to growth factors to augment MSC chondrogenesis, and manipulating scaffold microenvironment to recapitulate precartilage condensation and dynamic tissue morphogenesis

MSC chondrogenesis in tandem remains insufficiently characterized. It should also be recognized that the creation of a universal delivery platform to encompass the relevant biological, biochemical, and physical cues presents a significant technical challenge. Thus, the onus will be on the identification of vital elements from the vast scientific landscape and their optimal application to yield desirable outcomes. Furthermore, it would be crucial to determine the extent of in vitro priming necessary for optimal chondrogenic differentiation and neotissue development prior to implantation. Given that leveraging on such insights and their translation into medical practice has barely begun, developing GMP-grade materials and procedures will be the next frontier for successful clinical realization of functional articular cartilage repair.

Acknowledgements This work was supported by NUHS Bridging Funds. The authors thank Dr. Ying Ying Diao for her technical help in generating the SHG image. We wish to confirm that there are no known conflicts of interest associated with this publication.

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Single-Cell Analysis Approaches in Cartilage Diseases Diagnosis and Therapies

4

Mahsa Ghorbaninejad, Sara Farahi, Farzaneh Mirzaeian,
Fatemeh Khodabandehloo, Samaneh Hosseini,
and Mohamadreza Baghaban Eslaminejad

Abstract

Substantial evidence shows that major breakthrough toward cartilage therapeutic approaches is largely hampered by the low chondrocytes yield along with heterogeneous off-target differentiation of cells during chondrogenesis. Therefore, a complete assessment of an individual cell is essential for exhaustive comprehension of cell-to-cell variability. Nowadays, advances in stem cell biology have empowered the characterization of individual cells and biological mechanisms at the single-cell level. Single-cell analysis technologies represent the ultimate frontier of the transcriptomic landscape of thousands of single cells and

Mahsa Ghorbaninejad, Sara Farahi, Farzaneh Mirzaeian and Fatemeh Khodabandehloo contributed equally with all other contributors.

M. Ghorbaninejad · S. Farahi

Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

F. Mirzaeian

Stem Cell and Regenerative Medicine Group, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

F. Khodabandehloo

Department of Genetics and Advanced Medical Technology, Faculty of Medicine, AJA University of Medical Sciences, Tehran, Iran

S. Hosseini (✉) · M. Baghaban Eslaminejad (✉)

Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Department of Cell Engineering, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

e-mail: hosseini.samaneh@royaninstitute.org; eslami@royaninstitute.org

revolutionize our understanding of cartilage tissue during normal homeostasis and diseases. Novel technologies like microfluidics and CRISPR/Cas9 technology are valuable for single-cell omics analysis due to their sensitivity and accuracy. In this chapter, we first present the single-cell profiling of mesenchymal stem cell heterogeneity. Next, we discuss the recent progress in single-cell genomics, epigenomics, and proteomics sequencing technologies. The single-cell multi-omics approaches associated with genomics, transcriptomics, proteomics, as well as epigenomics are reviewed to identify cellular subpopulations that drive the disease. We also address their applications in biomarker discovery, personalized medicine, and regenerative medicine with the focus on cartilage tissue.

Keywords

Single-cell analysis · Single-cell multi-omics · Cartilage diseases

4.1 Introduction

Owing to the high degree of morphological complexity and avascular nature of articular cartilage, management of cartilage-related diseases is still a real clinical challenge. Accordingly, cell-based and biological therapies for cartilage diseases have emerged as promising research areas in regenerative medicine (Xiang et al. 2022). Single-cell sequencing technology has recently become the state-of-the-art method for uncovering the cellular heterogeneity and complexity, along with elucidating the composition of various cell types and their roles in highly structured tissues (Dong et al. 2022; Jovic et al. 2022). The single-cell analysis offers novel perspectives on biological questions that were not previously available in the fields of cartilage research. This technique is grouped by their targets and categorized as single-cell genomics, transcriptomics, proteomics, as well as epigenomics and metabolomics (Zhang et al. 2021; Chen et al. 2020). For the first time, in 1882, the banded structure of the chromosome was seen in the images of a single cell from the salivary gland (Flemming 1882). In 1990, a study used single hematopoietic cells to amplify the cDNA (Brady et al. 1990). Eberwine et al. designed a method to characterize single cells at a level beyond usual classifications and showed the gene expression pattern of living single cells in the rat hippocampus. The data showed that in spite of the same cell morphology, their gene expression profiles were different (Eberwine et al. 1992).

The single-cell RNA sequencing (scRNA-seq) analysis has offered a complete transcriptome landscape of heterogeneous populations which enable us to analyze the alterations at the single-cell level (Zhou et al. 2019). Single-cell RNA-seq methods provide powerful tools for understanding the transcriptome diversity, self-renewing dynamics, and differentiating stem cells as well as illustration of the

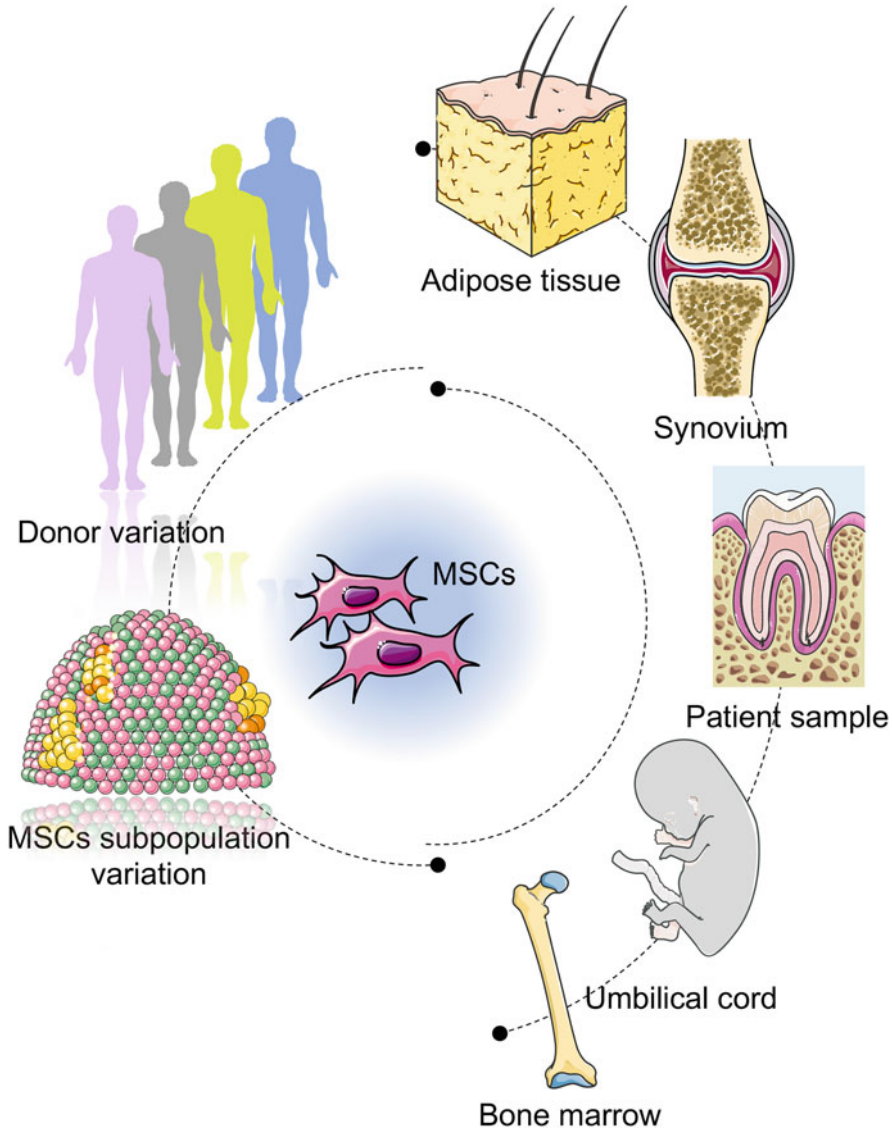


Fig. 4.1 Schematic representation of MSC heterogeneity. The heterogeneity of MSCs exists at three level including variable among the patient donor, tissue sources, and within MSC subpopulations

cartilage disease development and progression (Tang et al. 2009). Despite the remarkable therapeutic applications of MSCs in cartilage repair over the past two decades, their heterogeneity remains largely obscure. MSC heterogeneity arises from various factors including presence of various cell types in bone marrow, variations among the donors, batch-to-batch variations in culture medium, and culture conditions (Fig. 4.1). MSC subpopulations with a high potential for cartilage repair

are now being identified using a range of markers related to proliferation, differentiation, and inflammatory regulation. scRNA-seq as a novel technique has ability to determine MSC clustering with defined markers and assess the differences among MSC subsets (Zha et al. 2021). *Freeman* and colleagues used single-cell RNA-seq to reveal the transcriptional diversity of mouse bone marrow mesenchymal stem cells (mBMSCs). They found a high expression level of multipotency genes in MSCs. There were wide variations in the expression levels of chondrogenic genes among individual cells that did not belong to their proliferation status (Freeman et al. 2015). The potential of MSCs and their heterogeneity allows to select superior MSCs for the treatment of cartilage injuries. Single-cell technology is able to find disease biomarkers, cellular subsets, therapeutic targets, and diagnostics for cartilage-related disorders that would not have been detected by bulk sequencing.

The current chapter highlights the genomics, proteomics, and epigenomics platforms of the single-cell analysis approaches as well as single-cell multi-omics sequencing in cartilage tissue and diseases. We focus on the joint use of CRISPR/Cas9-based genetic screening and microfluidic single-cell omics analysis. The last part covers the single-cell analysis applications and discusses the impact of single-cell analysis in biomarker discovery, regenerative medicine, and personalized medicine.

4.2 Workflow in Single-Cell Technology

Single-cell technology workflow consists of three steps. In the first step, whole cells must be isolated. The advances in cell isolation methods have facilitated the ability of single-cell analysis in different areas including genomics, epigenomics, proteomics, transcriptomics, and multi omics.

Second step depending on the use of genomics or proteomics technologies is different. In single-cell genomics (SCG), the whole-genome amplification should be done. Whole-transcriptome amplification (WTA) and whole-genome amplification (WGA) are needed to produce basic materials for the sequencing library. DNA and RNA usually need to be amplified by WTA and WGA due to the minute amounts in a single cell.

The method for transcript detection of CEL-seq/CEL-seq 2, SCRB-seq, MARS-seq, Drop-seq, InDrop-seq, Sci-seq, SEQ-well, and SPLIT-seq is the 3'-end counting while for STRT-seq/STRT-seq2 is 5' only of transcripts. SMART-seq/SMART-seq 2, FISSEQ, and MATQ-seq can sequence the full length of transcripts (Paolillo et al. 2019). Another single-cell RNA sequencing method is Quartz-seq, which is suitable for identification of the heterogeneity in non-genetic cells related to the same tissue (Sasagawa et al. 2013).

Polymerase chain reaction (PCR) produces specific DNA fragments. Methods including primer extension preamplification PCR (PEP-PCR) (Zhang et al. 1992) and degenerating oligonucleotide-primed PCR (DOP-PCR) have been developed to amplify whole genomes (Cheung and Nelson 1996). Higher genomic coverage was also observed in multiple displacement amplification (MDA) than in the DOP-PCR method (Huang et al. 2015).

Deciding on selection methods (WTA and WGA methods) is based on the frequency of isolated single cells, the facility of RNA or DNA isolation from the desired cell, and the sequence coverage (depth) (Paolillo et al. 2019).

The final step is data analysis that includes processing and quality control (QC) for raw data, basic data analysis, and advanced analytics for specially designed studies (Su et al. 2022).

4.2.1 Single-Cell Isolation Technologies

Based on the available platforms, there are two different strategies for single-cell isolation. The first one is based on physical properties like density, size, electrical changes, and deformation, and the second one is based on biological properties (Kalisky and Quake 2011).

Preparing a single-cell suspension for musculoskeletal tissues, cartilage, tendons, and bone is problematic. It is more complicated in pathological conditions because of the tense and denseness of these tissues. Enzymatic methods are conventionally used to harvest single cells from these tissues. More recently, a tissue-specific enzyme cocktail has been developed to efficiently isolate qualified single cells from degenerated nucleus pulposus (NP), knee articular cartilage (AC), and ossifying posterior longitudinal ligament (OPLL) (Gao et al. 2022). Despite satisfactory results obtained from NP and OPLL, this approach must be improved for AC due to excessive cell aggregates and low viable cell counts.

The technologies used frequently for single-cell isolation are fluorescence-activated cell sorting (FACS), magnetic-activated cell sorting (MACS), laser microdissection, manual cell picking/micromanipulation, and microfluidics/lab-on-a-chip which the most important of these technologies are discussed below.

4.2.1.1 Fluorescence-Activated Cell Sorting (FACS)

FACS is a single-cell analysis method in a high-throughput fashion. This method is not appropriate for the isolation of very rare cells and environmental samples that contain very heterogeneous cells (Saliba et al. 2014). The need for high quantity cell population, destruction of cell function, and architecture because of its suspension are drawbacks to this method.

4.2.1.2 Laser Capture Microdissection (LCM)

LCM is a powerful technique used for isolating single cells or cell portions from solid tissue (Emmert-Buck et al. 1996; Espina et al. 2007). The microdissection system enables the dissection of a wide variety of living tissue. LCM also makes it possible to extract live cells for the culture (Golubeva et al. 2012). Coupling LCM with RNA-seq helps to identify glioblastoma heterogeneity and intra-tumoral differences in distinct histological compartments (Civita et al. 2019).

4.2.1.3 Microfluidics

Microfluidics has revolutionized our knowledge of biological processes by increasing the potency of single-cell resolution. There are three essential microfluidics-based technologies for the separation of the single-cell including trap-based microfluidics, valve-based microfluidics (lowest throughput), and droplet-based microfluidics (highest throughput) (Gross et al. 2015; Gómez-Sjöberg et al. 2007; Brouzes et al. 2009; Edd et al. 2008). The droplet- and microwell-based microfluidics promoted the development of scRNA-seq (Pan et al. 2022). For example, in a study by Stephenson et al. droplet-based single-cell transcriptomic profiling was performed on five rheumatoid arthritis patients. They extended a low-cost and 3D-printed droplet microfluidic control tool. Thirteen transcriptomically distinct subpopulations were obtained from single-cell sequencing by this technique. The new fibroblast subpopulations were recognized and also their spatial distribution within the synovium was determined (Stephenson et al. 2018).

4.2.1.4 Magnetic-Activated Cell Sorting (MACS)

MACS is one of high-throughput affinity-based isolation methods commonly used in single-cell analysis approaches. In this technique, specific cell surface proteins are labeled by matched antibodies, enzymes, lectins, or streptavidins conjugated to magnetic beads. After labeling, cells will be isolated by flowing in the magnetic field which polarizes the cells of interest, while other cells are discarded (Welzel et al. 2015).

4.3 Various Type of Single-Cell Technologies

After decades of efforts, high-throughput-based technologies were broadened to analyze the heterogeneity of the cells in order to discover each individual cell state and diversities relevant to diagnosis and therapeutics approaches. Single-cell analysis methods are grouped by their targets and categorized as single-cell genomics, epigenomics, transcriptomics, proteomics, and metabolomics which are described in detail in the following.

4.3.1 Single-Cell Genomics (SCG)

In recent years, SCG has been developed to simultaneously measure the expression level of genes in a large number of single cells. This approach is a powerful tool for studying cell heterogeneity and rare cells. There probably is genetic heterogeneity within identical cell types in a tissue. A major challenge that still needs to be addressed is understanding the small differences at the level of genomes and transcriptomes that give rise to distinct functions that cannot be detected by usual methods.

The idea of genome analysis at the single-cell resolution is not an idea of recent years. Before the development of single-cell technology, bulk-tissue next-generation sequencing (NGS) technology has been used for genome transcription and sequencing. The first single-cell transcriptome was performed in 2009 using a next-generation sequencing platform. They identified unknown splice junctions only with a single mouse blastomere that were not detected by microarray (Tang et al. 2009). In 2011, Islam et al. presented a single-cell tagged reverse transcription (STRT) that is a *multiplexed scRNA-seq* (Islam et al. 2011). Later, the mRNA-Seq protocol (Smart-Seq) was designed by Ramsköld et al. for single-cell transcriptome analysis. This protocol helps to identify single nucleotide polymorphisms (SNP) and improves the read coverage of transcripts (Ramsköld et al. 2012). Cell expression by linear amplification and sequencing (CEL-Seq) extended by samples pooling and barcoding before linearly amplifying mRNA using a round of in vitro transcription. This method is more sensitive and repeatable compared to amplification methods based on PCR (Hashimshony et al. 2012).

Single-cell genome sequencing helps to identify variations in the level of chromosome including copy number and single nucleotide, revealing the changes related to genomic heterogeneity (Hu et al. 2016). In 2014, Zong et al. used multiple annealing and looping-based amplification cycles (MALBAC) as a new amplification method for full-length amplifying of the transcript. This method has uniformity throughout the genome. MALBAC amplified single-cell DNA of SW480 cancer cells with the coverage over 93% (Zong et al. 2012). Smart-Seq was improved for capturing full-length transcripts and also promoted in precision and sensitivity. Smart-seq2 was introduced with a precious protocol for the production of full-length cDNA and having a library for sequencing (Picelli et al. 2014).

4.3.2 Single-Cell RNA Sequencing

Single-cell RNA sequencing approach improved over time; starts with sequencing a small number of cells, but over time, it has increased to millions of cells simultaneously. Single-cell digital gene expression profiling in blastomere was performed by Tang et al. in 1990 which discovered many transcripts that were overlooked by that time. Single-cell transcriptomics provides novel insights to study heterogeneity of the single cells (Adil et al. 2021).

The research in the field of single cell has influenced diagnoses and therapy of diseases such as immunology, tumor heterogeneity, prenatal genetic diagnosis, neuroscience, and cell differentiation. Due to the lack of cartilage intrinsic capacity for self-repair and insufficient expression of certain markers, recognition of the internal state of the chondrocyte is essential. A research group investigated the gene expression profile in chondrocytes isolated from patients with osteoarthritis (OA) at different stages at single-cell resolution. They identified novel markers related to chondrocytes, signaling pathways involved in the osteoarthritis pathogenesis, and novel subgroups of chondrocyte on the basis of scRNA-seq. In order to achieve early diagnosis and treatment of OA, the relationship between the

transcriptome scheme and the clinical output of the disease was found. In this study, the researchers revolved new strategies for managing and diagnosing arthritis to improve health care and lifestyle (Ji et al. 2019).

Single-cell sequencing *can be used* in the pathogenesis of cartilage tissue to identify the heterogeneity data between different cell populations in order *to understand* the connection between cell type and diseases and consequently disease diagnosis and treatment. This strategy has the power to report *differences* in *expression level* of protein and genetic material at the single-cell level. *Early diagnosis and treatment* achieved by this approach would reduce *joint pain* caused by cartilage diseases and the costs imposed on family and society. Bulk RNA sequencing and single-cell RNA sequencing were also combined to investigate the regulatory genes induced chondrogenesis in human-induced pluripotent stem cells (hiPSCs). In addition, dynamic transcriptomic profiles involved in the differentiation and proliferation of chondrocytes were determined (De Kinderen et al. 2022). Another research group conducted a study to reveal the relationship between diversity in a specific cell type and differentiation by using scRNA-seq. They determined gene signatures and cell-specific subsets in healthy individuals and patients with degenerated meniscal cells and found altered proportion of clusters in degenerated meniscal cells compared to those of healthy individuals. They also identified a cluster specific to meniscal degeneration with the characteristics of progenitor cells. Identifying new mechanisms of meniscal degeneration can help to strengthen therapeutic strategies and methods for meniscal repair (Sun et al. 2020).

A scRNA-seq on knee articular cartilage and menisci of humans revealed that there is a common pathogenic cell population between the two tissues. The expression profile of this cell population has been seen in osteoarthritis and senescence. The expression of genes involved in OA pathogenesis, including senescence, has been promoted by fibroblast-activating protein (FAP) and transcription factor ZEB1 (Swahn et al. 2022). Recently, scRNA-seq analysis of degenerated human meniscus and healthy samples has shown microenvironmental changes in the inner and outer meniscus during degeneration and identified the previously uncharacterized chondrocyte subpopulations related to degeneration as a new therapeutic aim (Fu et al. 2022).

Xiaoyu Li et al. *combined the analysis of bulk RNA-seq and scRNA-seq data* during OA progression and recognized key genes and gene expression patterns. Furthermore, transcription factor and long non-coding RNA (lncRNA) regulation were identified. The results of this study showed lncRNA-mediated regulation between NRP1 and CYTOR could be related to the vascularization of cartilage and pain in *osteoarthritis of the knee* (Li et al. 2021).

Regarding intervertebral disk (IVD) disease (IDD), many *transcription factors* and *molecules* involved in the retention of NP and annulus fibrosus (AF) are not well-defined. An unbiased scRNA-seq approach on a nucleus isolated from IVD patients was performed to define each segment by identifying the gene expression profile. Gene enrichment analysis showed the expression of different genes in NP and AF. The results of functional annotation clustering revealed the upregulation of distinct gene pathways related to AF and NP which reflects their specific function. In

addition, due to the uniqueness of AF and NP transcription factors, it is suggested that these factors play an crucial role in regulating transcriptome signatures of IVD (Fernandes et al. 2020).

Ferroptosis is a new form of programmed cell death (nonapoptotic) by the iron-dependent accumulation of lipids. In a study to investigate the action mechanism of ferroptosis resistance in senescent chondrocytes (SenChos), single-cell RNA sequencing and metabolomics were performed. Targeting the membrane protein excitatory amino acid transporter protein 1 (EAAT1) in osteoarthritis induces ferroptosis which causes the selective cleaning of SenChos. As a result, the progression of osteoarthritis slows down (Wen et al. 2023).

4.3.2.1 Single-Cell Spatial Transcriptomics

The next step in the analyze the single cell can be spatial single-cell transcriptomics. The function of every cell in the tissue can be related to the spatial location of that cell (Marx 2021). Important spatial information can help to understand the cell-to-cell relationship in diseased and healthy individuals. The combination of spatial transcriptomics and scRNA-seq can distinguish the characteristics of single cells in terms of transcriptional specificity in their own place (Longo et al. 2021).

4.3.3 Single-Cell Proteomics

Among the single-cell analysis approaches, single-cell proteomics is more challenging due to protein abundance and the complexity of the proteome. Besides, since there is no extension method for (similar to PCR for DNA amplification) the noise reduction, protein detection assays must be extremely sensitive with high multiplexing capacity to minimize technical errors and detect the negligible concentration range of proteins. Key roles of proteins in the biological process such as cell signaling, migration, vesicular traffics and secretion, differentiation, cell division, and apoptosis reveal the importance of identifying proteins in disease and treatment studies. Moreover, DNA and RNA analysis cannot entirely reflect the protein expression level due to post-transcriptional/translational regulation systems in cells. Single-cell protein quantification methods are classified as **immunoassay techniques**, **mass spectrometry tools**, and **microfluidics-based platforms**. Referring to the crucial role of bottom-up proteomics in biological and clinical research, the advanced methods in this area are reviewed in the following.

4.3.3.1 Immunoassay-Based Techniques

Immunoassay-based techniques uniquely identify the proteins involved in biological processes like cell identity, and behavior with high specificity through labeled antibodies. These methods included flow cytometry, mass cytometry, and imaging mass cytometry.

Flow cytometry is a semi-quantitative and the most frequent method for protein identification in the field of single-cell protein analysis. This method is designed on the basis of recording the fluorescence characteristics of single cells when they are

passing through the laser beam. Flow cytometry is a powerful toolkit for disease diagnosis and following up the treatment procedure. However, the fluorophore overlap limited this technique as well as cell loss in the sample preparation process (Giesen et al. 2014).

Mass cytometry or CyTOF (cytometry by time-of-flight mass spectrometry), a powerful tool in single-cell proteomics, is emerged by integrating flow cytometry and mass spectrometry techniques and introduced by Ornatsky et al. (2008). CyTOF overwheals the bottleneck of spillover errors and the limited analysis ability of protein numbers in flow cytometry by applying metal-labeled antibodies instead of fluorophores. Simultaneous analysis of more than 40 isotopes per cell has been reported in the CyTOF method while only up to 20 protein parameter detection is possible in flow cytometry (Williams et al. 2020). However, the slow acquisition rate and lack of cell-collecting features in comparison with flow cytometry are the flaws.

Imaging mass cytometry (IMC) is a new method for single-cell protein detection which arises from the mass cytometry platform to visualize proteins on a slide that is scanned sequentially in the mean of illustrating the complete image. Like CyTOF, IMC has also a slow rate of processing, and an enormous amount of data is an issue in this method (Zhang and Vertes 2018; Grandi et al. 2020).

4.3.3.2 Mass Spectrometry Tools

Antibody-based methods are restricted to high-quality antibody accessibility and are intrinsically limited in their multiplexing capacity. Moreover, targeting cells needs to have good knowledge about different cells in a cell population which is not possible in all cases. To overcome these challenges, mass spectrometry approaches have entered proteomics. Single-cell mass spectrometry (scMS) provides large-scale quantitative analysis of proteins in a label-free and untargeted manner. Mass spectrometry techniques are primarily based on liquid chromatography (LC) integrated with electrospray ionization (ESI) (Sahu et al. 2022). These approaches are classified into two categories: first, vacuum-based ion sources, including secondary ion MS (SIMS), matrix-assisted laser desorption ionization (MALDI)-MS, and matrix-free laser/desorption/ionization (LDI)-MS methods with high sensitivity. Second, ambient ionization methods encompass electrospray ionization-ion mobility spectrometry (ESI-IMS)-MS, laser desorption ionization droplet delivery (LDIDD), laser ablation electrospray ionization (LAESI), and atmospheric pressure MALDI (AP-MALDI) approaches, with the potential of profiling proteins in living cells. The main drawbacks of implementing MS are the cost of the instrument and big data analysis (Liu and Singh 2013).

Single-cell proteomics has the potential to be applied in clinical applications for both diagnosis and therapeutic tasks. Providing the proteome maps for each individual cell by single-cell protein profiling methods assists in the early diagnosis of disease and personalized treatments. Powerful proteomics methods are expanded to discover protein networks in various disorders like cartilage and chondrocyte disease. Since cartilage injuries and degeneration are unrepairable, early diagnosis is critical. Among cartilage disorders, OA is the most prevalent disorder that causes

cartilage degeneration and chronic pain. *Fiorella Carla Grandi* et al. investigated (Shema et al. 2016) different cell signaling states, implying single-cell mass cytometry tools to study both the pro-regenerative cell populations and the inflammatory populations in patients with OA. In another study, *Neety Sahu* and her colleagues (Shema et al. 2019) used the single-cell mass cytometry technique to follow the effects of preclinical drugs on cartilage homeostasis. Despite the importance of using single-cell-based protein analysis for cartilage disease, only a few reports are available in the literature, which may be related to the fact that proteomics technology is in its infancy phase; however, the growth rate is remarkable.

4.3.3.3 Microfluidics-Based Platforms

The miniaturization, provided by microfluidics technology, has developed rapidly in single-cell profiling methods through extending existing technologies like Western blot, flow cytometry, and ELISA. Microfluidics is a powerful platform capable of being implemented in small volumes of samples and utilizing different sample preparation methods and varied workflows in a single device. The small scale of microfluidic chips led to a quick buffer exchange and minimized reagent and cell requirements to reduce cost with the ability to investigate a small amount of cells from a patient's biopsy samples. Moreover, the miniaturized scale provides high control ability in a stable cellular microenvironment. All these together, microfluidics afford fast acquisition processing with acceptable sensitivity in single-cell proteomics. The current technologies utilized in microfluidic methods are listed here as microfluidic fluorescent flow cytometry, microwell-based assay (microengraving), droplet-based microfluidics, microchamber-based assay (barcoding microchips), single-cell Western blotting, and microfluidic single-cell ELISA. All these methods are growing fast in single-cell protein profiling as well as other high-throughput technologies (Clark et al. 2006).

4.3.4 Single-Cell Epigenomics

Epigenetics focuses on the study of genome regulatory principles in the nucleus. These controlling systems maintain the diversity within the cells, which are genotypically identical. Traditional epigenomic tools utilized bulk assessment of chromatin modifications in a cell population, which did not actually mirror the correlation between epigenetics and molecular scale mechanisms for every single cell. For instance, H3K4me3 represents active transcription while H3K27me3 is associated with gene repression. Since the bivalent histone modification (activating and suppressing) on a promoter may occur for the same or different histones simultaneously or even different alleles and cells, the bulk analysis is not able to clarify the real event of these single molecules progress, whereas single-cell resolution potentially recognizes the true molecular mechanisms for an individual cell (Miura et al. 2012). Therefore, during the last decades, single-cell-based methods have arisen and grown very quickly to develop a better insight into epigenetics and chromosome remodeling in various types of cells. Single-cell epigenomic (SCE)

technology potentially plays an essential role in identifying biological processes, signaling networks, drug discovery, disease diagnosis, and therapeutic approaches. Additionally, single-cell methods are able to analyze the cell state even the low number of cells derived from patients.

The functional output of the genome is controlled by various epigenetic marks like DNA methylation, histone modification, and chromatin topology. Therefore, the epigenomic state of a single cell requires to be investigation in different dimensions, encompassing DNA methylation, histone modification, chromatin accessibility, nucleosome positioning, and chromosomal contacts. Each of these states is reviewed via various methods, which are discussed in the following.

4.3.4.1 Single-Cell DNA Methylation Profiling

DNA methylation plays a critical role in regulating gene expression involved in biological processes such as development, differentiation, and gene imprinting. CpG islands are the main targets of methyl transferase enzymes to create 5-methylcytosine (5mC) in mammals. Since abnormal DNA methylation is proven in many cancers and complex disorders, analyzing DNA methylation sites provides a robust monitoring approach for disease pro/diagnosis and treatment responses (Lorthongpanich et al. 2013).

Profiling methylated regions through epigenetic tools is well adapted to single-cell resolution, and a variety of assays are established based on the methylation class, which includes 5mC and less abundant derivatives like 5hmC, 5fC, and 5caC. Bisulfite-based sequencing (scBS-seq), which is the first popular epigenomics single-cell approach for detecting 5mC, is known as the gold standard in terms of profiling DNA methylation level. Bisulfite is a chemical reagent that changes unmethylated cytosine to uracil, while methylated cytosines remain unconverted (Zhu et al. 2017). However, DNA degradation following bisulfite treatment has limited this method. Therefore, to increase the scalability of single-cell methylation profiling, it is suggested to perform bisulfite conversion before library preparation, which is called the post-bisulfite adapter tagging (PBAT) technique (Rotem et al. 2015) or alternate the bisulfite chemicals with methylation-sensitive restriction enzymes, which are known as single-cell restriction analysis of methylation (SCRAM) (Rhee and Pugh 2012).

Technically, bisulfite sequencing and restriction enzyme-based protocols are not able to distinguish between 5mC and less abundant 5hmC, so to probe rarer versions of methylated derivatives, other protocols have been developed. For this purpose, restriction endonucleases AbaS1 in scAba-sequencing and CpG methylase M.SssI in methylase-assisted bisulfite sequencing (MABseq) utilize the restriction endonucleases to identify the glucosylated 5hmC and 5fC, respectively. Besides, chemical-labeling-enabled C-to-T conversion sequencing (CLEVER-seq) is another chemical approach to detect 5fC (Snyder et al. 2016).

4.3.4.2 Single-Cell Histone Modification Mapping

Histones are principal components of chromatin that regulate gene expression, based on internal or external signaling. Hence, histone mapping at a single-cell scale

reveals gene regulatory mechanisms in cell function as well as in disease study and therapeutic approaches. Chromatin immunoprecipitation sequencing (ChIP-seq) is the first established method for epigenome-wide analysis of histone marks this context is based on histone-specific antibodies. ChIP-seq at the single-cell level is restricted due to false positives and disturbance noise relating to nonspecific antibodies (Kelsey et al. 2017). To overcome this challenge, it is suggested to use pooling data on related loci or perform higher resolution techniques like ChIP-exo and ChIP-nexus, which in the exonucleases are used to digest the protein-bound DNA to generate the fragments with 25–50 base pairs, which are then mapped by sequencing approaches (Nagano et al. 2013).

4.3.4.3 Single-Cell Chromatin Conformational Assessments

Open chromatin regions (OCRs) are naked nucleotide areas in the genome that are controlled by DNA regulatory elements. Several studies have addressed the attribution of OCRs to the mechanisms that end up with disease and cell abnormalities (Reid et al. 2017). Epigenomic approaches decode the correlation between ORCs and gene expression via established methods adapted to single-cell resolution. The first method is DNase-sequencing, which is designed based on the sensitivity of OCRs to the DNase I enzyme and adjusted for single-cell assays. Nevertheless, the low-throughput scalability and nonspecific signals are drawbacks of this protocol. To achieve higher-throughput platforms for chromatin accessibility mapping, the assay for transposase-accessible chromatin sequencing (ATAC-seq) technique is developed, in which Tn5 transposase is able to add adapters into ORCs but not the covered regions by the nucleosomes; consequently, the inserted markers lead to identifying the OCRs (van Meurs et al. 2019) and TF patterns in each individual cell.

In addition to the linear chromatin organization, the higher-order conformation of chromatin can be captured at the single-cell level via the Hi-C-based method. Chromatin topology features a concerted chromatin interaction network. Hi-C technology is used to understand a wide range of interactions within genome elements. The Hi-C workflow starts with DNA–protein complex fixation, digestion via sequencing restriction enzymes, random ligation, and fragment amplification by PCR. Finally, deep sequencing platforms carry out base-pair resolution of the ligated fragments. High-throughput scalability and the ability to detect long-range DNA interactions make Hi-C a powerful experimental tool on the single-cell scale (Angermueller et al. 2016).

The development of single-cell-based technologies has an intensive impact on disclosing the heterogeneity and rare subpopulations of cells in a tissue, which provides an exciting opportunity to develop our vision for molecular mechanisms involved in diseases and design novel treatment strategies. Different studies have proved that many diseases are related to genome-wide or loci-specific alteration of DNA methylation, histone modification pattern, and aberrant chromatin organization (Cao et al. 2018). Therefore, a large number of bulk-sample epigenomics assessments have been performed to reveal the epigenetic variation in healthy versus diseased cartilage (Zhu et al. 2019). Using approaches like chromatin structure sequencing, DNA methylome sequencing, and histone modification analysis on a

single-cell scale helps researchers to achieve greater knowledge about the function of epigenetic regulations in the cartilage diseases, however, the single-cell resolution has not been reported yet.

4.3.5 Single-Cell Multi-Omics

Multi-omics as a high-throughput approach poises the correlation of genomics, transcriptomics, proteomics, and epigenomics, which afford a comprehensive understanding of cellular events. Multi-omics platforms are advanced in various dimensions, including parallel profiling of the DNA methylome and the transcriptome (Lee et al. 2020), chromatin accessibility and the transcriptome (Christopher et al. 2022; Stein et al. 2021), and the genome and transcriptome at single-cell resolution. The sub-categories of these approaches are classified into three various layers, as the follows:

4.3.5.1 Single-Cell Genome and Transcriptome

Genome/transcriptome profiling techniques are now well adapted to single-cell approaches. These platforms can be categorized into two groups. The first one includes the methods based on the early cytoplasmic RNA and genomic DNA separation which is finally analyzed by scRNA sequencing and wide genome sequencing tool like genome and transcriptome sequencing (G & T-seq), simultaneous isolation of genomic DNA and total RNA (SIDR), and single-cell triple omics sequencing (scTrio-seq). While in the second group, the primary separation phase is not performed as gDNA and RNA separation occurred at the sequencing steps like gDNA-mRNA sequencing (DR-seq) and TARGET sequencing. The current methods can reveal the association of genomics alteration and gene transcription patterns in diseases and therapeutic pathways (Tang et al. 2019).

4.3.5.2 Single-Cell Proteome and Transcriptome

The correlation between transcription and functional protein states can be determined by high-throughput multi-omics protocols. Technically, proteome and transcriptome protocols are based on scRNA sequencing and antibody screening. They can probe cell surface proteins like cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), and RNA expression and protein sequencing assay (REAP-seq) platforms or detect intracellular proteins like RNA and immunodetection (RAID), proximity extension assay/specific RNA target amplification (PEA/STA), and proximity ligation assay for RNA (PLAYR) methods. These approaches provide a clear insight into the heterogeneity in post-transcriptional and translational regulations which cannot be followed by proteomics or transcriptomics alone (Soul et al. 2018).

4.3.5.3 Single-Cell Epigenome and Transcriptome

DNA methylation, histone modification, and chromatin conformational changes play key roles in controlling gene expression. Parallel analysis of epigenome and

transcriptome extremely assists to understand the regulatory events within cells, driven by internal and external cell signaling. Among these protocols, both single-cell methylome and transcriptome sequencing (scM & T-seq) and single-cell triple omics sequencing (scTrio-seq) focus on methylation assessment and RNA sequencing. However, single-cell combinatorial indexing of chromatin accessibility and mRNA (sci-CAR), and single-nucleus chromatin accessibility and mRNA expression sequencing (SNARE-seq) methods measure the open chromatin region (OCRs) and mRNA levels. The next approach is the single-cell nucleosome, methylation, and transcription sequencing (scNMT-seq) which is able to simultaneously profile chromatin accessibility, DNA methylation, and transcriptome for an individual cell. Moreover, joint analysis of Chip-seq and RNA-seq provides a comprehensive insight into the relationship between transcription factors and RNA levels. However, Chip-seq protocols have not yet expanded to multi-omics assays.

In general, the obtained data from single-cell multi-omics integration techniques potentially identify the global molecular networks in a single cell from a cell population, which has an intensive impact on clarifying complex disease mechanisms and pathophysiology. These techniques are widely used in cancer research; however, no studies have been reported on cartilage disorders yet (Tang et al. 2019) (Fig. 4.2).

Among the complex diseases, OA has a remarkable prevalence among people with an average age of 70 years. Complex diseases like OA are driven by both environmental and genetic factors. Many bulk multi-omics studies have been developed to discover the molecular mechanisms and cellular events in OA. However, the single-cell-based studies have yet to be reported.

Since cartilage degeneration and localized inflammation are interfering with OA, multi-omics methods in single-cell resolution can reveal mechanisms underlying osteoarthritis across several molecular layers.

4.4 Single-Cell Analysis Applications

Single-cell analysis approaches are currently being applied to elucidate the various developmental stages as well as the development of multicellular organisms. Basically, single-cell analysis opens a new era in biomarker discovery, regeneration medicine, and personalized medicine in stem cell therapy for cartilage defects (Stein et al. 2021). This technique has become one of the most indispensable tools for dissecting cellular heterogeneity and identifying cell types and/or cell states.

4.4.1 Biomarker Discovery

Biomarker discovery referred to a measurable indicator of normal biological processes, as well as pathogenic processes of the disease stages. It has many potential applications in diagnosis, prognosis, and monitoring of disease progression, as well as pharmacodynamic and dose-response studies. The challenges of healthy and

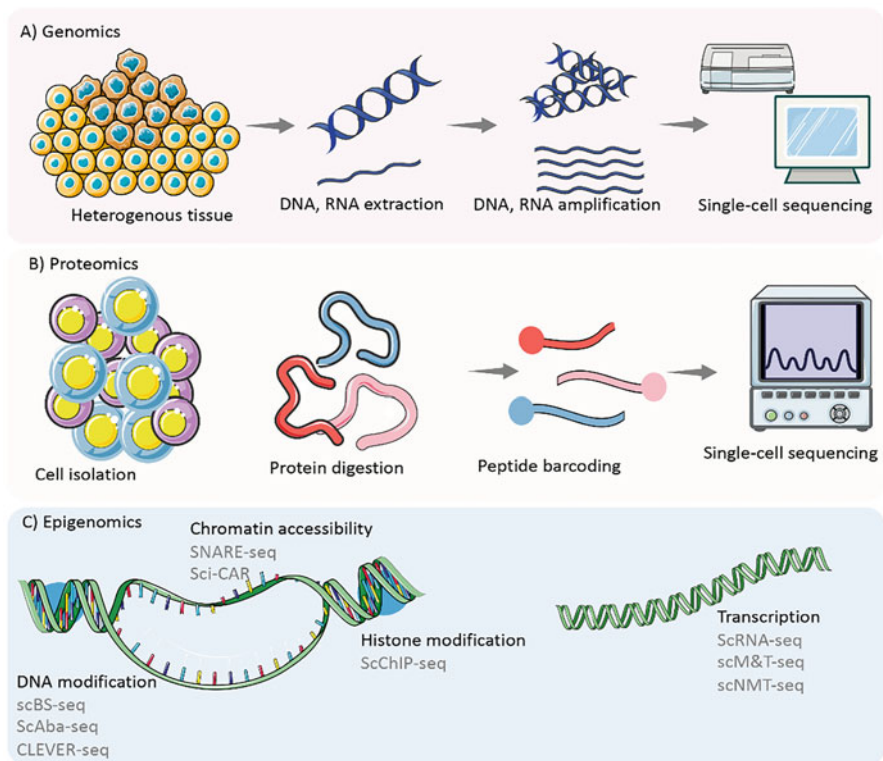


Fig. 4.2 Single-cell analysis methods are categorized as single-cell genomics, proteomics, and epigenomics. (a) Genomics is a process of isolating DNA and RNA from heterogeneous tissue, followed by nucleic acid amplification, single-cell sequencing, and data analysis. (b) Proteomics consists of several steps, including the extraction of the protein mixture from the sample, followed by peptide barcoding and single-cell analysis, and (c) different technical epigenomics approaches, including chromatin accessibility, DNA methylation and modification, histone modification, and transcriptome techniques, are shown

diseased tissue heterogeneity hindered biomarker discovery and development studies. During the past decade, clinical and transcriptional profiling studies dramatically increased to find biomarkers, risk factors, pathways, and targets of cartilage diseases (Sahu et al. 2022). Nowadays, analyzing single cell is widely applicable for characterization of heterogeneity in cartilage-related diseases to find effective biomarkers and drug (Tang et al. 2019). Tan et al. for the first time, used single-cell RNA sequencing (scRNA-seq) to investigate whole-transcriptome complexity in individual cells (Tang et al. 2009). Currently, developing scRNA-seq as one of the high-throughput tools plays a crucial role in both medicine and basic research.

There is an unmet need to know more about the molecular mechanisms behind cartilage degeneration and regeneration in osteoarthritis (OA) in order to improve therapeutic strategies. In this context, Soul and colleagues performed RNA sequencing (RNA-Seq) of knee cartilage of patients with OA and healthy people in order to

identify patient subgroups and compare major pathogenic pathways. The results of this work revealed two subgroups of OA supporting the presence of two major pathogenic pathways. Also, PhenomeExpress analysis were applied to investigate the differences between the two subgroups of OA. The two subgroups were different in innate immune responses, as well as altered Wnt and transforming growth factor beta (TGF- β) signaling, while a notable absence of inflammatory cytokine activation was observed at both of them. In addition, a total of 478 biomarkers were predicted in synovial fluid based on gene expression to distinguish patients between the two subgroups (Soul et al. 2018). In another study, Ji et al. performed single-cell RNA-seq on the articular cartilage obtained from 10 patients with OA. The novel definition for the sample severity index of OA articular cartilage was used to evaluate the relationships between chondrocytes from OA cartilage and the available clinical data. Seven molecularly defined populations were elucidated in 1464 chondrocytes of human OA at different stages with three distinct function phenotypes. Cartilage progenitor cell-related markers and their association with fibrocartilage chondrocytes were determined using computational analysis. This high-throughput analysis clarified various cell type function for early diagnosis and treatment of OA (Ji et al. 2019).

The pathogenesis of disease onset and progression is crucial to clarified the hub genes and signaling pathways. Recently, Jiang and colleagues have illustrated that LINC00167 in peripheral blood leukocytes may be a potential diagnostic biomarker of OA. Also, a total of 18 candidate Lnc-RNAs were found after first-stage validation. According to this study, LINC00167 expression in serum could be a reliable diagnostic marker for patients suffering OA (Jiang et al. 2021).

High-throughput molecular screening revealed the differentially expressed mRNA transcripts between preserved allograft and fresh cartilage. The expression levels of ECM-related mRNAs were reduced in the stored allografts (Lin et al. 2016). Whole-genome RNA sequencing revealed differences between transcriptomic profiles of Femoroacetabular impingement (FAI) and hip OA patients. Among the total of 3531 differentially expressed genes (DEGs) between the OA and FAI cohorts, the expression level of FGF18 and WNT16 significantly upregulated in the FAI samples. FGF18 expression were also increased in OARSI grade 1–2 FAI samples compared to OA and OARSI grade 5–6 FAI samples. This negative association of FGF18 expression in FAI and hip OA patients suggested that FGF18 signaling may be used as a biomarker for the progression of FAI-induced OA (Kuhns et al. 2022).

Despite the considerable advances in the discovery of new biomarkers for cartilage-related diseases, it is still at an early stage. There are some challenges to overcome including non-invasive diagnosis and prognosis biomarkers, replacement of liquid biopsy instead of conventional tissue, and improvement of novel detection technologies. In addition, there are very few single-cell analyses which are available on cartilage-related biomarker discovery that may reflect the novelty of these techniques and necessity of expertise to execute the experiments and analyze data (Fig. 4.3).

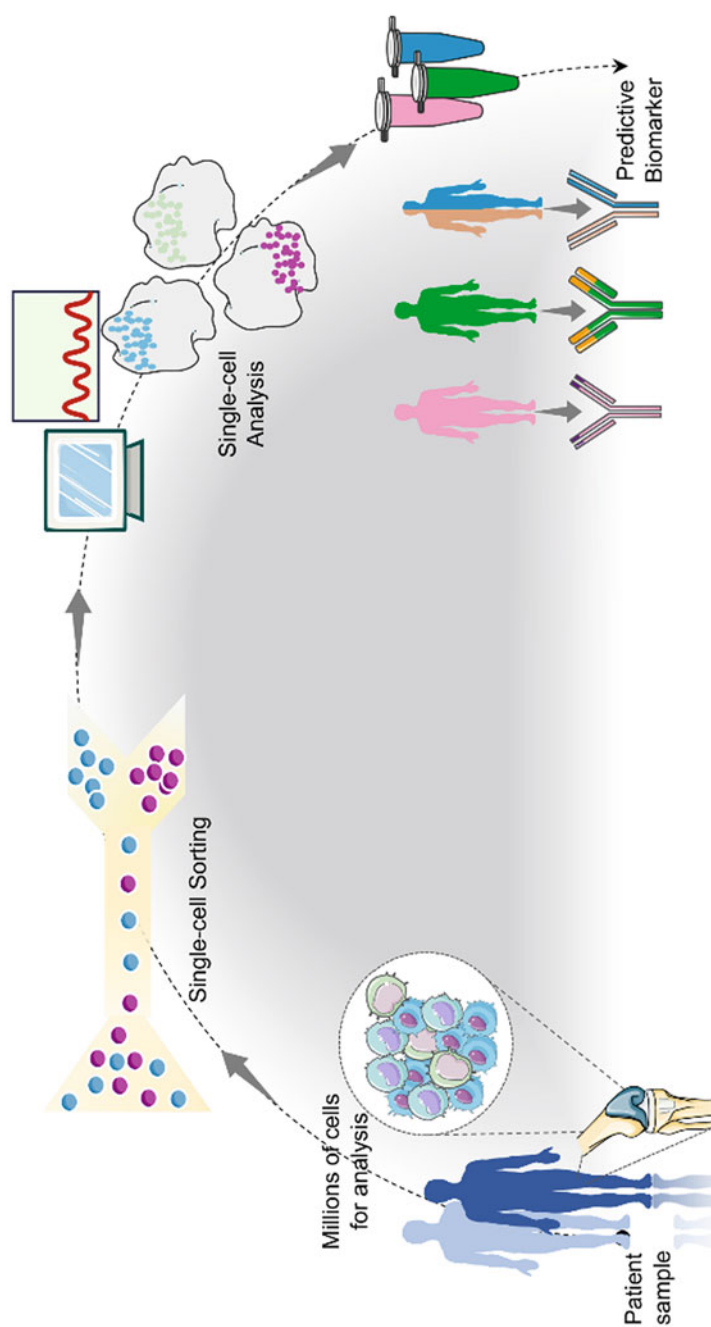


Fig. 4.3 An overview of single-cell application in biomarker discovery. Single-cell analysis to biomarker discovery can be summarized in the four steps: sample collection of patients, sorting of single cells based on functional properties, single-cell analysis and statistical analyses, and biomarker discovery of the mechanism at a single-cell level

4.4.2 Single-Cell Genomics and Regenerative Medicine

Regenerative medicine focuses on the replacement of dead or dysfunctional cells, to repair or regenerate tissues and organs. Therefore, in recent years, many studies have been conducted on stem cell transplantation and tissue regeneration for the treatment of diseases.

4.4.2.1 Evaluation of Accuracy and Precision in Regenerative Medicine and Tissue Engineering

In order to create a disease model or even regenerate damaged tissues, pluripotent stem cells (PSCs) can be differentiated into the desired cells in a 2D culture, through the forced expression of transcription factors which define cell type. Furthermore, PSCs may produce organoids by organizing into complex 3D structures that closely resemble natural 3D body tissue. However, there are many challenges in the use of stem cells in tissue engineering and regenerative medicine. For example, the differentiation potential of stem cells is affected by factors such as tissue source and isolation method. This uncertainty is also true for organoids as it is obvious that many native tissue cells do not exist in the 3D environment of organoids. Indeed, when stem cells differentiate, it is possible for them to differentiate into the desired cells but not exactly like a real cell. The term “accuracy” (A proportion of the *in vitro* transcriptome that is identical to the correlated primary cell) refers to this degree of similarity. It is also possible that the differentiated cell is completely similar to the cell but of a non-target lineage. The ratio of the target to the off-target engineered cell line is defined by the term “precision” (Zhang et al. 2020). Therefore, accuracy and precision are important for success in tissue engineering.

Single-cell RNA sequencing (scRNA-seq) is more precise at the gene expression level compared to whole-genome sequencing, which provides information about a certain group of cells or the most dominant cells. For example, by using scRNA-seq, the researchers were able to find the reason for this problem, and why the differentiation of human-induced pluripotent stem cells (hiPSCs) into chondrocytes followed by cartilage regeneration is of low efficiency (Han et al. 2022). Unbiased information provided by single-cell analysis of cellular heterogeneity at multiple time points during hiPSC differentiation indicated that WNTs and MTF, as specific hub genes, drive off-target differentiation to neural cells and melanocytes. Finally, by targeting WNTs and MTF, they remove off-target cell lineages and considerably increased the function and homogeneity of hiPSC-derived chondrocytes (Han et al. 2022). This study provides a high-resolution strategy to realize the transcriptome disposition during lineage differentiation.

4.4.2.2 Single-Cell Atlases as a Reference for Tissue Regeneration

By using high-throughput scRNA-seq technologies, it became possible to produce cell atlases from a large number of mouse and human tissues. Therefore, we could prepare a molecular profile of each type of cell in each organ and tissue, at different times of development and across different individuals. The atlas obtained from the

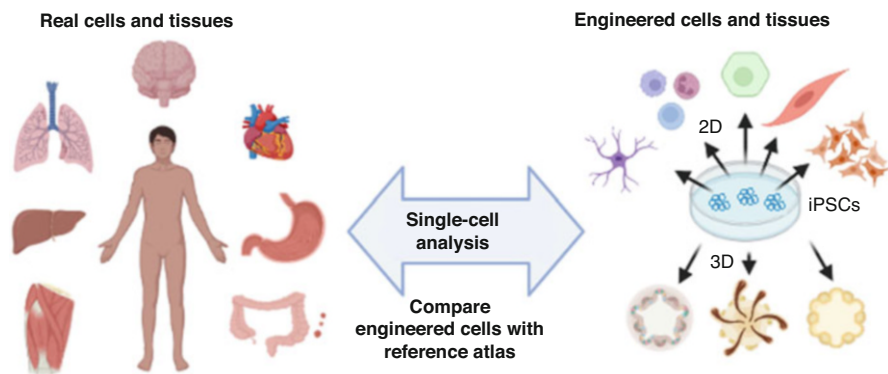


Fig. 4.4 A cell atlas derived from single-cell sequencing as an unbiased reference for cell and tissue engineering

scRNA-seq results can serve as a reference for quantitative comparison between engineered and naïve cells (Fig. 4.4).

Moreover, by comparing the transcriptional profile of cells in different stages of the development of a disease with reference cells, it is possible to discover formerly unknown cell populations, as well as cell type-specific transcriptional profiles and molecular pathways that contribute to the disease progression (De Kinderen et al. 2022; Chou et al. 2020; Sebastian et al. 2021; Chang et al. 2021; Chaudhry et al. 2022; Seidl et al. 2019). For example, *Ji et al.* aimed to understand the mechanism of cartilage degeneration and regeneration in osteoarthritis (OA) and using scRNA-seq identified seven articular chondrocytes populations in this disease. They also found some changes associated with OA progression (De Kinderen et al. 2022). Single-cell transcriptome analysis of 10,640 synoviocytes and 26,192 chondrocytes was also demonstrated the the molecular cross-talk between synovium and cartilage and in OA (Chaudhry et al. 2022). In another study, *Sebastian et al.* determined distinct molecular profiles of chondrocytes harvested from intact and damaged joints (Seidl et al. 2019). Using single-cell transcriptome profiling before and after traumatic injury, they identified 9 transcriptionally distinct chondrocyte subpopulations in the mouse knee joints. They also found that the molecular changes occurred in these subpopulations within 7 days after the trauma. In summary, defining the initial molecular changes induced by injury can provide new therapeutic targets for the treatment of cartilage degeneration.

4.4.2.3 scRNA-seq-Related Methodologies to Guide Cell and Tissue Engineering

Transcription Factor Combination

By using the scRNA-seq technique and applying the reverse engineering process, we are able to identify a combination of transcription factors that exist in specific cell types and subgroups, as well as at branch points along the differentiation pathway.

Therefore, the obtained results make it possible to improve the efficiency of reprogramming or differentiation into a desired cell line and evaluate the exact role of external stimuli in differentiation and regeneration. As an example, *Chang et al.* used single-cell RNA sequencing analysis and found that electrical stimulation by affecting the metabolism, biogenesis, and change of calcium ion in the micromass of canine adipose tissue-derived mesenchymal stem cells (ADSCs) led to the development of prechondrogenic cell aggregation and eventually enhanced the production of proteoglycan and reduced the expression of collagen type 1 in stimulated cells (*Liang et al.* 2022).

Spatial Reconstruction

Many cellular signals and pathways occur in specific spatial and temporal locations in a complex tissue. Therefore, it is important to analyze the behavior of cells in their native niche. Although the scRNA-seq technique disturbs the spatial tissue integrity, combination of scRNA-seq technique with emerging approaches, for instance sequential single-molecule fluorescence in situ hybridization, enables us to evaluate the spatiotemporal states of the cell.

CRISPR-Cas9 System

Different studies have used the CRISPR-Cas9 technique to knock out the target genes and evaluate their effect on the differentiation of chondrocytes and cartilage regeneration, as well as the progression of OA (*Kriks et al.* 2011; *Basad et al.* 2015; *Bao et al.* 2021). Therefore, the use of CRISPR screening with scRNA-seq would be helpful to identify the effect of genetic alterations in each individual cell and the genes that are essential for the appearance of specific subtypes. Furthermore, this technique makes it possible identify barriers that lead to inefficient cell differentiation and unexpected fates.

4.4.2.4 Importance of scRNA-Seq in Disease Modeling and Therapy

One of the most important applications of iPSCs is to create a disease model by applying desired mutations using CRISPR-Cas9 and differentiation of iPSCs into target cells or tissues. By using scRNA-seq, we are able to identify the distinct effects of these mutations on various cells grown in a heterogeneous 2D cell cultures or 3D organoids, and search for disease-associated phenotypes in all subpopulations.

Although many cell transplant treatments are in the clinical trial stage (*Lin et al.* 2022; *Lu et al.* 2020), one of the limitations of this method is batch-to-batch variations and the different responses to treatment. The scRNA-seq combined with cell transplantation therapies enable scientists to evaluate the purity, safety, and heterogeneity of each batch and identify biomarkers for quality control.

4.4.3 Single-Cell Sequencing and Personalized Medicine

Personalized medicine has attracted more attention following the advances in approaches that enhanced our knowledge about the molecular basis of the disease,

especially genomics. In this field of medical science, individual's genetic profiles are used to make more accurate decisions about the prevention, diagnosis, and treatment of diseases. Here, we emphasize the potential of single-cell sequencing in personalized medicine in cancers including chondrosarcoma. However, these results should be generally applicable to other complex diseases such as OA.

4.4.3.1 Dissection of the Tumor Microenvironment

The term tumor microenvironment (TME) refers to the interaction of tumor cells, surrounding non-cancerous cells and non-cellular parts of the extracellular matrix. Tumor cells interact with non-cancerous cells and non-cellular compounds through complex signaling pathways and use them for their own benefit. The consequence of these interactions is cancer recurrence, incomplete response to treatment, and drug resistance. It should be noted that the type of cells in the TME can be different in various cancers or in patients with the same type of cancer. Therefore, accurate and unbiased evaluation of TME by high-throughput techniques such as single-cell sequencing would help to better understand the tumor-promoting microenvironment and signaling pathways, as well as identify biomarkers for disease prognosis. For example, *Bao et al.* used scRNA-seq and showed that M2-like tumor-associated macrophages (M2-TAMs) were the main tumor-infiltrating immune cells, which might lead to poor prognosis in breast cancer through immunosuppression (*Wang et al. 2020*). TME investigation in laryngeal chondrosarcoma by scRNA-seq identified 5 types of cells including chondrocytes (the most common cell type), myeloid cells, lymphocytes, as well as fibroblasts and endothelial cells in the tumor microenvironment (*Miyamoto et al. 2015*). It was also found that the SLAMF9 gene was specifically expressed in chondrosarcoma non-immune cells, while it was slightly expressed in adjacent intact cartilage tissues. Therefore, this study has provided clues to identify the mechanisms involved in TME heterogeneity.

4.4.3.2 Tumor Heterogeneity Assessment

Cancer is a complex and highly heterogeneous disease at the genetic, epigenetic, and phenotypic levels. Heterogeneity can be related to cells within the same tumor (intra-tumor heterogeneity) or between different metastatic tumors in a single patient (inter-tumor heterogeneity). Greater tumor heterogeneity can lead to poor response to therapy, drug resistance, higher metastasis, and poor overall survival. Therefore, successful treatment would be achieved by understanding and evaluation of the heterogeneity of the tumors. A research group compared two types of lung adenocarcinoma including ground glass nodule (GGN) and solid adenocarcinoma (SADC) using scRNA-seq (*Bodenmiller et al. 2012*). This study showed that angiogenesis signaling pathways were downregulated, collagen expression by fibroblasts was reduced, and immune cells were more activated in GGN. These findings have provided useful insight to better explain the prognosis of GGN patients compared to SADC patients.

4.4.3.3 Study of Therapy Resistance

Therapy resistance is a major issue in the treatment of cancer. Single-cell sequencing provides identification of low-frequency mutations that can lead to drug resistance and gain a better view of drug resistance drivers (Fig. 4.5). Bone marrow single-cell sequencing in a patient with drug-resistant MCL (mantle cell lymphoma) revealed 10 subpopulations that included 4 malignant B cell subtypes, 3T cell subtypes, 2 dendritic cell subtypes, and 1 natural killer (NK) cell subtype. It was found that three main mechanisms involved in treatment resistance were anti-perforin activity, inhibition of apoptosis, and reduced immune response which were mediated by type I and II B cells (Wang et al. 2020). Miyamoto et al. studied the mechanism of drug response in prostate cancer at the single-cell level. scRNA-seq of circulating tumor cells (CTCs) showed that most patients who were resistant to androgen deprivation treatment had one or more types of androgen receptor mutation or splicing variants in their CTCs. In addition, patients who were treated with enzalutamide (second-line androgen receptor inhibitor) due to drug resistance had CTCs with activated non-canonical Wnt signaling (Miyamoto et al. 2015). Therefore, single-cell analysis of CTC shows heterogeneity in signaling pathways that can contribute to treatment failure.

4.4.3.4 Drug Development

Drug development is an expensive and time-consuming process with a high failure rate. By using single-cell analysis, a more accurate and comprehensive picture of genetic, epigenetic, and phenotypic events can be obtained in drug responders

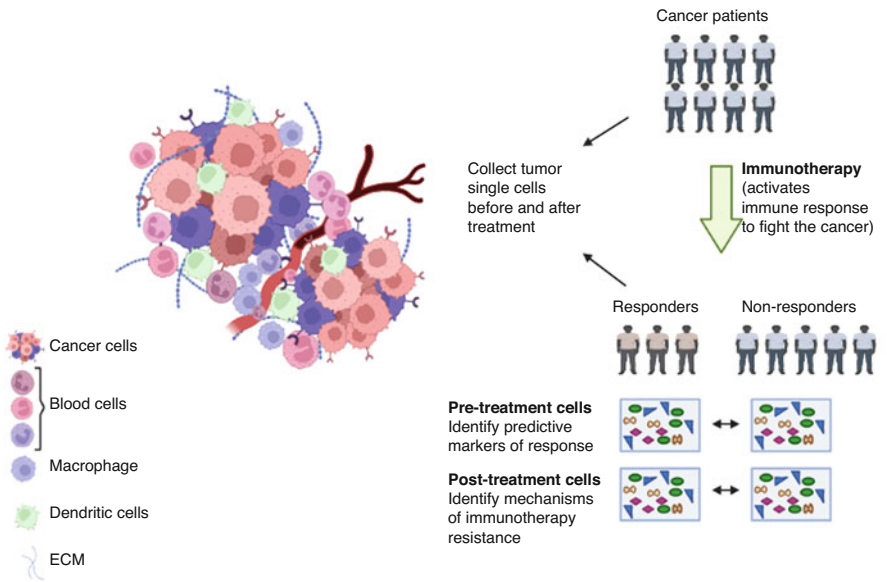


Fig. 4.5 Single-cell analysis to identify therapy resistance mechanisms

compared to non-responders at single-cell level. Thus, drug resistance, drug targets, and drug reactions and toxicities can be more accurately identified. Using cellular barcoding, a large number of samples can be analyzed simultaneously. For instance, during mass cytometry, cells are divided within a multi-well plate and marked by distinctive combination barcodes that specify a certain cell with its position in the well (Bodenmiller et al. 2012).

4.5 Concluding Remarks and Future Perspective

Simple, automated, and fast commercial single-cell sequencing platforms enable diagnostic applications at the single-cell level. However, to make most of this technique, obstacles such as computational skills for data analysis, the lack of consensus protocols for preparation of single-cell suspensions from a tissue, and data interpretation should be overcome. Since molecular heterogeneity has been observed in complex cartilage diseases including OA (Seidl et al. 2019), more studies are necessary to investigate the impact of these heterogeneities on the treatment of different patients. It is hoped that with the progress of these studies, the possibility of more effective personalized treatment for patients will be provided.

Considering the promising role of MSCs in cartilage repair, the heterogeneity of MSCs has posed a challenge to the development of regenerative medicine. Understanding heterogeneity as a fundamental characteristic of MSCs is a critical step in efficiency of MSC-based strategies. Over recent years, advances in understanding the single-cell analysis technique have greatly expanded our knowledge about the molecular mechanism involved in MSC subsets during chondrogenic differentiation as well as cartilage tissue. The single-cell genomics could investigate the genomic characteristics of the chondrogenesis as well as whole-genome de novo mutation rates. Although single-cell proteomics has limited detection sensitivity due to the easy loss of proteins during processing, single-cell protein profiling methods provide the proteome maps of each individual cell and may exert beneficial in early diagnosis of cartilage-related diseases. Transcriptional regulation of different epigenetic layers can be investigated subsequently using epigenome single cells. Single-cell analyses have the potential to prepare microenvironments well suited for biomarker discovery. Furthermore, single-cell analysis has the potential for therapeutic discovery for personalized medicine as well as regenerative medicine. In the near future, the rapid development of single-cell multi-omics approaches would improve our understanding of the molecular pathogenesis involved in cartilage-related diseases and open new insights into the human development of diseases. Despite the critical role of this technology in clinical diagnosis and therapeutic applications, there are limitations that should be overcome to ameliorate both the content and the quality of the information obtained from single-cell multi-omics analysis.

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The Importance of Mechanical Stimulation in Cartilage Formation: Applications of Bioreactors

5

Kaihu Li, Valentina Basoli, Zhen Li, and Sibylle Grad

Abstract

Articular cartilage in native joints is subject to a complex mechanical milieu in terms of load and motion patterns. Biomechanical stimuli play a key role in cartilage formation and maintenance of cartilage homeostasis. In cartilage tissue engineering, it is favorable to implement bioreactors, which are devices that can exert biomechanical loadings in vitro. Cartilage-specific bioreactors simulate native mechanical stimuli to favor neo-tissue formation. In this chapter, we first introduce the biochemical and biomechanical characteristics of articular cartilage, and highlight the properties, developments, and applications of bioreactors in cartilage tissue engineering. Then, the underlying mechanisms of mechanotransduction in chondrogenic cells are addressed. Furthermore, new perspectives for mechanically stimulated cartilage models are illustrated.

Keywords

Articular Cartilage · Bioreactor · Mechanotransduction

K. Li

AO Research Institute Davos, Davos, Switzerland

Department of Orthopaedics, Xiangya Hospital of Central South University, Changsha, China

Department of Orthopaedics, The Second Xiangya Hospital of Central South University, Changsha, China

V. Basoli · Z. Li · S. Grad (✉)

AO Research Institute Davos, Davos, Switzerland

e-mail: sibylle.grad@aofoundation.org

5.1 Mechanical Functions and Properties of Articular Cartilage

Cartilage is predominantly composed of 65–80% water (Sophia Fox et al. 2009), around 25% collagen, and approximately 5% proteoglycans (PGs) in its wet weight (Mollenhauer 2008). Collagens are a group of structural proteins in the extracellular matrix (ECM) which have one or more domains with a triple helix and non-helical regions at both ends of the helix (Knupp and Squire 2003). Collagen is an important determinant of the mechanical properties of cartilage. PGs are comprised of a core protein with attached sulfated glycosaminoglycan (GAG) side chains. The major PG in cartilage is aggrecan. Aggrecan has a “bottle-brush” structure comprised of chondroitin sulfate and keratan sulfate side chains attached to a core protein with multiple functional domains. The PGs are dispersed along the collagen fibers and oriented in the same direction as the collagen fibers (Mollenhauer 2008; Vernengo et al. 2020). Some minor PGs, like decorin and fibromodulin, are localized in the interterritorial matrix between cells, whereas biglycan has a pericellular distribution (Oldberg 1993). Epiphykan has been identified in the ECM surrounding resting, proliferating, and hypertrophic chondrocytes (Johnson et al. 1999). Versican is also found in the territorial zone immediately surrounding cell lacunae (Matsumoto et al. 2006).

Articular cartilage provides a smooth, lubricated surface for diarthrodial joints with a low frictional coefficient and facilitates the transmission of loads to the underlying subchondral bone (Sophia Fox et al. 2009). The unique mechanical properties of cartilage are defined by its specific zonal structure and ECM composition in each zone. The superficial zone is primarily composed of collagen fibers (type I, type II, and type IX) (Sophia Fox et al. 2009; LaPrade et al. 2008). The fibers are oriented parallel to the articular surface, imparting tensile and shear properties to cartilage (Vernengo et al. 2020). Concordantly, the chondrocytes in this zone are flattened and oriented parallel with the fibers, secreting superficial zone protein (SZP), also known as lubricin or proteoglycan 4 (PRG4), which facilitates lubrication of the surface by reducing friction (Peng et al. 2016). The middle zone of cartilage is composed of collagen II and aggrecan. In the middle zone, the collagen fibers are thicker than in the superficial zone and arranged in a random orientation, providing compressive resistance to cartilage (Vernengo et al. 2020). The deep zone contains the highest amount of PGs. The collagen fibers here have the largest diameter and are arranged in a columnar orientation, perpendicular to the cartilage surface. Consequently, this zone of cartilage has the highest compressive strength (Vernengo et al. 2020). There is a low density of spherical chondrocytes in the middle and deep zones. The tidemark marks the transition to the calcified cartilage and is identifiable histologically by a differing staining pattern than the middle and deep zones. The calcified zone is a stiff mineralized tissue that serves as the junction to the subchondral bone (Mollenhauer 2008). Here, the collagen fibers, composed of collagen type II, type IX, and type X, are directly connected to subchondral bone (Vernengo et al. 2020).

The mechanical properties of articular cartilage are defined by its components in 2 phases: (1) a fluid phase containing water and inorganic ions and (2) a solid phase containing the porous and slightly permeable ECM (Sophia Fox et al. 2009; Mow

et al. 1984). The highly negatively charged PGs and interstitial fluid provide compressive resilience to cartilage through negative electrostatic repulsion forces (Sophia Fox et al. 2009). Mechanical loading applied on the cartilage induces an increase in interstitial fluid pressure, which generates a frictional drag on the ECM (Sophia Fox et al. 2009; Mow et al. 1984; Frank and Grodzinsky 1987; Maroudas and Bullough 1968). This flow-dependent viscoelastic behavior provides important load support, which reduces the stress acting upon the solid ECM (Mow et al. 1984; Hayes and Mockros 1971). Therefore, for evaluation of the mechanical properties of cartilage native tissue and tissue-engineered constructs, the most common parameters characterized are compressive stiffness modulus and frictional properties (Patel et al. 2019). Compressive tangent modulus can be calculated from a ramp test, with a constant compressive strain applied until a specified strain rate is reached. The reported tangent modulus can vary more than 100,000 times depending on the selected compressive strain rate and at which strain the modulus is calculated. Single-step and multiple-step stress relaxation test is another commonly used mechanical test method to calculate the equilibrium modulus of cartilage. This method shows a much better output consistency compared with the ramp test, with a cartilage equilibrium modulus ranging around 150–300 kPa under unconfined compression (Patel et al. 2019). The frictional properties which reflect the effectiveness of cartilage tissue lubrication can be measured by migrating contact area test or stationary contact area test. Again, here the output has a large variation and would depend on the selected testing method.

Biomechanical stimuli play an important role in cartilage development and maintenance of cartilage homeostasis (Berendsen and Olsen 2015; Fahy et al. 2018). Mechanical forces regulate the formation of the growth plate through Indian hedgehog (IHH) and parathyroid hormone-related peptide (PTHrP) signaling pathways (Ng et al. 2006; Wu et al. 2001; Wu and Chen 2000). Shear stress and hydrostatic pressure have been postulated to modulate cartilage ossification (Carter et al. 1987; Simon 1978). The thickness of formed cartilage tissue is related to mechanical loading (Beaupre et al. 2000). Dynamic compressive loading has shown to enhance the expression levels of transforming growth factor beta 1 (TGF- β 1) in chondrocytes, which reduces hypertrophic differentiation and mineralization (Ohno et al. 2005). Mechanical loading within a physiological range is essential for the maintenance of homeostasis in healthy cartilage. It is well documented that mechanical forces stimulate cartilage ECM synthesis, such as PGs, collagen type II and IX, and cartilage oligomeric matrix protein (COMP) (Mauck et al. 2000; Ng et al. 2009). Mechanical loading is also beneficial for suppression of pro-inflammatory genes (Dossumentkova et al. 2007).

Age-related pathological changes have been shown to lead to osteoarthritis (OA) with mechanical property changes to cartilage (Loeser et al. 2016). During OA progression, oxidative stress leads to accumulation of reactive oxygen species (ROS) in the cartilage ECM and there is an overproduction of proteolytic enzymes causing a loss of essential aggrecan (Buckwalter et al. 1994; Hui et al. 2016). The levels of small PGs also change with age. For instance, decorin expression is increased in aged human articular cartilage (Melching and Roughley 1989).

Fibromodulin and lumican were shown to be susceptible to degradative proteinases in OA (Shu et al. 2019). There is an accumulation of advanced glycation end products (AGEs) in the collagen fibers (Verzijl et al. 2002; Vos et al. 2012). All the changes lead to alterations in the mechanical properties, such as increased compressive stiffness and loss of reversible deformation (Sophia Fox et al. 2009).

In tissue engineering strategies for repair and regeneration of articular cartilage, mechanical cues are also essential factors for the differentiation and maturation of chondrocytes or mesenchymal stem cells (MSCs). The effect of mechanical stimulation on chondrocytes (Anderson and Johnstone 2017) and MSC chondrogenesis (Fahy et al. 2018; Choi et al. 2018) has been well summarized in several recent reviews. Sophisticated bioreactors have been developed to apply dynamic mechanical regimes on tissue-engineered cartilage constructs. Mechanical loading shows favorable effects on cartilage ECM synthesis, mechanical properties, and cellular phenotype regulation. The type of loading, including compression, shear, and hydrostatic pressure, influences the outcome of the engineered tissue (Salinas et al. 2018). For each loading condition, the duration, frequency, and amplitude need to be optimized depending on the properties of the constructs.

5.2 Mechanical Milieu of Articular Cartilage

In vivo the articular joints are subject to complex load and motion patterns during daily activity. Thereby the cartilage is exposed to multiple types of loads, including static and dynamic compression, shear, fluid flow, and hydrostatic pressure. Intact hyaline cartilage is capable of enduring thousands of compressive loading cycles without any restriction. Physiologically, the reported dynamic compressive stresses acting on the articular cartilage during normal daily movement reach around 5–8 MPa, as measured in human hip joints (Afoke et al. 1987; von Eisenhart et al. 1999). Similar data were documented from human knee joints, where stresses reach 5–6 MPa during walking. Intermittent lower loads down to 0.5 MPa have also been observed in load-bearing joints (Mow and Wang 1999). Peak contact stresses reaching up to 18 MPa, when rising from a chair, were measured in an instrumented human hip (Hodge et al. 1989) endoprosthesis. Also, during activities such as climbing stairs, pressures can reach 10–20 MPa in the human knee (Morrell et al. 2005).

Physiological loading is beneficial for maintaining the homeostasis of the healthy joint, while abnormal loading is detrimental for the turnover of the cartilage. Aberrant loading can occur due to mechanical trauma, chronic overloading, joint instability, or obesity; however, immobilization and subsequent lack of mechanical stimuli (Arden and Nevitt 2006) also have negative consequences for cartilage integrity and function. One of the underlying reasons for the cartilage breakdown after excessive mechanical load is chondrocyte apoptosis. Stresses of more than 10 MPa have been shown to induce apoptosis in articular chondrocytes (Kerin et al. 1998); a study on bovine cartilage explants found that loads of 4.5 MPa were already

sufficient to trigger an apoptotic response, while degradation of the collagen fiber network occurred at high stresses of 7–12 MPa (Loening et al. 2000).

In terms of dynamic loading frequency, a frequency of approximately 1 Hz has typically been considered as normal for human gait, whereby values of more than 2 Hz are super-physiological. The range of cartilage deformation or strain during *in vivo* loading has been determined by imaging techniques. The deformation depends on both the anatomical location within the joint and the extent of the activity. In addition, these compressive strains are not equal throughout the depth of the cartilage. In the superficial zone, >50% of the strain is experienced, an average strain can be attributed to the transitional zone, while <5% is experienced by the deep zone cartilage (Eckstein et al. 1999). A magnetic resonance imaging (MRI) examination (Mosher et al. 2005) showed that after a period of 20 min of running, the deformation of the knee joint cartilage was about 20% in the weight-bearing areas of the femoral side, whereas strains of up to 30% were noted in the tibial areas. During normal walking, deformations in the range of 7–23% were measured in tibiofemoral cartilage of human subjects (Liu et al. 2010); thereby, the strains were highest in the medial areas of the knee joint. Due to the viscoelastic nature of the hyaline cartilage and its slow recovery, the deformations may accumulate during the day; this results in a reduction in cartilage thickness at the end of the day. In the human knee, these diurnal variations in thickness again strongly depend on the location (Coleman et al. 2013). While no significant diurnal difference in cartilage height was measured in some areas like the femoral groove, the thickness changed by 11% in the medial tibia. However, it needs to be considered that physiological strains are changing in cases of cartilage injury or disease. For example, diurnal cartilage strains significantly increase in subjects with a high body mass index (Widmyer et al. 2013). Also, injury of the anterior cruciate ligament, joint instability, or aging can change the experienced strain and hence contribute to cartilage degeneration (Bischof et al. 2010; Van de Velde et al. 2009).

Hydrostatic pressure is experienced by articular chondrocytes as a result of the substantial water content within hyaline cartilage. During joint loading, water is pressed out of the tissue, while during subsequent unloading, the high concentration of negatively charged GAGs leads to the absorption of abundant water molecules. Under normal movement conditions, the joint cartilage is exposed to hydrostatic pressures ranging from 0.5 to 10 MPa (AufderHeide and Athanasiou 2004; Elder and Athanasiou 2009). These physiological hydrostatic pressures contribute to the maintenance of cartilage homeostasis, although both anabolic and catabolic responses to hydrostatic pressure *in vitro* have been reported (Hodder et al. 2020). In general, PG synthesis has been improved upon application of static hydrostatic pressures in the range of 5–10 MPa over an extended period of time. In both direct compression and hydrostatic pressure, excessive stress (>10 MPa) or strain (>20%) can have detrimental effects on the mechanical properties of cartilage. Thereby, injurious compression leads to a shift from anabolic to catabolic metabolism of chondrocytes (Quinn et al. 1998; Ashwell et al. 2013). In contrast, static loading or dynamic loading with unphysiologically low frequency can inhibit the synthesis of matrix components (Guilak et al. 1994).

Shear stresses are experienced by the cartilage surface during joint motion. Low-to-moderate shear is physiological for the intact cartilage surface but can be detrimental if the tissue is damaged. Friction that is higher than in healthy condition, e.g., due to trauma or degeneration, increases the shear stress at the cartilage surface. This can initiate catabolic reactions, resulting in further degradation as a vicious cycle (Lin and Klein 2021). Under physiological conditions, and assuming a compressive pressure of 1 MPa, an approximate shear strain of 1% has been estimated (Lin and Klein 2021). Both direct and fluid-induced oscillatory shear occur during joint articulation. In addition, shear load improves the nutrient perfusion, leading to an indirect positive effect on cartilage turnover. The extent of shear stress varies greatly between different locations within the joint. It has been observed that in areas of high shear stress, such as the femoral condyles, the thickness of the superficial zone was increased compared to areas of high load-bearing and low shear stress (Arokoski et al. 1999). Importantly, shear load is a major stimulus of PRG4, or lubricin, expression, and secretion, contributing to the maintenance of a low friction articular surface (Nugent et al. 2006; Grad et al. 2005). The therapeutic effect of post-operative continuous passive motion (CPM) may thus primarily be based on the influence of such shear-induced stimuli (Knapik et al. 2013).

5.3 Development of Bioreactor Culture Systems

In the areas of tissue engineering and preclinical model development, a “bioreactor” can broadly be described as a system that controls the biochemical and/or biomechanical environment of cells or tissues *in vitro*. The use of bioreactors has become increasingly popular in musculoskeletal research, whereby devices that apply mechanical stimuli play a primary role (Peroglio et al. 2018). For cartilage tissue engineering, bioreactors have early been introduced to modulate cell growth, phenotype, and activity and to promote cartilaginous ECM formation. Initially, basic types of bioreactors were applied for seeding and culturing of cells within scaffolds for tissue engineering purposes. These bioreactors included spinner flasks, rotating wall vessels, and perfusion systems. The main function of the bioreactor was to improve the diffusion of oxygen, nutrients, and waste products into and out of the scaffold. Furthermore, the applied mechanical stimuli facilitated the homogenous distribution of cells and matrix molecules within the scaffold (Freed et al. 1993).

Spinner flask cultures contain cell-seeded scaffolds suspended in the medium or held in place by a vertical needle-type holding system. The medium is circulated within the flask using a stirring bar. An early study showed that rabbit articular chondrocytes cultured in spinner flasks better maintained their chondrogenic phenotype, producing more collagen type II and GAGs, compared to cells grown in monolayer culture (Norby et al. 1977). The implementation of spinner flasks for culture of chondrocytes in three-dimensional (3D) scaffolds in general improved the collagen II and GAG synthesis, which was mainly attributed to the improved nutrient and gas exchange, while the stirring intensity had a minor effect (Gooch et al. 2001). Later, spinner flasks were used to grow and culture articular

chondrocytes in microporous scaffolds or hydrogels. The main emphasis was placed on the enhancement of cell expansion and the maintenance of the chondrogenic phenotype (Schrobback et al. 2011).

Also, the effect of spinner flask cultivation on the differentiation of MSCs was investigated. For example, spheroid formation and chondrogenic differentiation of human adipose tissue-derived stromal cells were induced by culturing the cells in spinner flask suspension bioreactors (Yoon et al. 2012). Similarly, dynamic culture significantly enhanced the generation of cartilaginous constructs in silk–fibroin–chitosan scaffolds seeded with umbilical cord blood-derived human MSCs (Agrawal et al. 2018), which was demonstrated by GAG quantification, histology, immunofluorescence, and expression of chondrogenic genes. Nevertheless, the use of spinner flasks has limitations with respect to nutrient exchange in larger constructs. Furthermore, the occurrence of excessive shear forces may induce adverse effects on cell activity. Finally, shear stresses are known to promote osteogenesis which could result in aberrant differentiation, especially if MSC differentiation toward the chondrogenic lineage is desired.

Rotating wall vessels were described in the 1990s as bioreactors that simulate microgravity conditions (Freed and Vunjak-Novakovic 1995). Hereby, the culture medium is moved in a circulatory manner. During rotation of the vessel, the cell-seeded constructs are exposed to a constant movement producing vector-averaged forces comparable to those of reduced gravity or free fall. These vessels were used for seeding of chondrocytes into polymer scaffolds and 3D culturing of the constructs. It was demonstrated that culture of chondrocytes under simulated microgravity resulted in improved GAG accumulation compared to solid body rotation in rotating bioreactors, turbulent mixing in spinner flasks, and orbital mixing in petri dishes (Freed and Vunjak-Novakovic 1997; Vunjak-Novakovic et al. 1999).

Interestingly, a comparative study using human bone marrow-derived MSCs (BMSCs) cultured in chondrogenic or osteogenic differentiation medium under spinner flask and rotating wall vessel conditions showed beneficial effects of spinner flask culture on both chondrogenic and osteogenic marker expression under their respective media conditions (Wang et al. 2009). On the other hand, the rotating wall vessel culture did not seem to promote MSC differentiation compared to the static culture controls. In contrast, another study revealed beneficial effects of microgravity culture of BMSCs on the GAG per DNA synthesis compared to pellet cultures (Sakai et al. 2009). Overall, these bioreactors focus primarily on media mixing, while applying certain shear stresses on the outer part of the scaffold. Moreover, in rotating wall vessels, the effects of unloading on cellular growth and phenotype can be studied.

Unidirectional and oscillating perfusion were later found to yield tissue-engineered constructs with improved structure and mechanical properties. Under perfusion culture, the experienced stresses can be better controlled. Thereby a porous scaffold is press-fit into the chamber and directly perfused with medium. Perfusion bioreactors have been effectively used for cell seeding and culturing. In MSCs, such stimuli can induce cell proliferation and chondrogenic differentiation (Zhao and Ma 2005; Alves da Silva et al. 2011). Both the increased nutrient transport and the shear

stress influence the cellular response independently (Li et al. 2009). Indeed, bioreactors that generate shear stresses have been used more frequently to induce osteogenic rather than chondrogenic differentiation. Several studies demonstrated the beneficial effect of perfusion culture on osteogenic differentiation of MSCs. Nevertheless, chondrogenic differentiation can also be promoted by shear stresses (Schulz and Bader 2007; Zuscik et al. 2008). Therefore, it is essential to consider the combined effects of the cellular environment, including the biomaterial properties, medium composition, oxygen tension, and the mechanical influence, when optimizing the differentiation of MSCs toward the desired lineage. To monitor cell differentiation, the gene expression ratio of Runx2/Sox9 can be determined as an early indication of MSC fate (Loebel et al. 2015). In general, the spinner flasks, rotating wall vessel, and perfusion bioreactor systems may be beneficial for 3D chondrocyte or MSC growth and maintenance, and for assessing the impact of distinct shear forces and patterns on the cell phenotype. Interestingly, perfusion culture appeared to promote chondrogenic and subsequent hypertrophic differentiation of BMSCs *in vitro*, although this did not result in improved bone formation in a critical-size femoral defect in nude rats *in vivo* (Bernhard et al. 2018). Finally, careful evaluation of the applied stimuli is essential to find the optimal parameters that keep the balance between the effect of mass transport and the chondrogenic or osteogenic shear stress levels. For example, flow rates between 0.01 and 0.1 mL/min were reported to enhance cell growth and viability, while higher flow rates (0.2 mL/min) were effective to induce osteogenesis (Hadida and Marchat 2020).

5.4 Cartilage-Specific Bioreactors Applying Mechanical Stimulation to Favor Neotissue Formation

Since mechanical stimulation plays an important role in *in vivo* articular cartilage development and maintenance, bioreactors, as *in vitro* culture systems, have been developed to simulate native biomechanical loadings as well as biochemical conditions. Bioreactor studies demonstrated that biomimetic stimuli have significant effects on cartilage formation, maintenance, and the delay of OA onset (Schulz and Bader 2007; O'Connor et al. 2013). For *in vitro* cartilage tissue engineering, the goal of exogenous mechanical loads applied by bioreactors is to ultimately promote chondrocyte anabolic activity and ECM generation. To generate cartilaginous tissue *in vitro*, both chondrocytes and MSCs have been widely used. According to the experimental objectives, culture systems could use monolayer culture, 3D scaffold-free biomass, 3D cell-laden constructs, or cartilage explants.

Types of mechanical loadings that can be mimicked by bioreactors include compression, shear stress, hydrostatic pressure, and multiaxial loading (Fig. 5.1). During the loading process in bioreactors, the settings of mechanical parameters include the loading type, magnitude, frequency, pressurization type, loading time, application period, and timing of application. In addition, regulation of non-mechanical factors in bioreactors can also influence *in vitro* chondrogenesis and ECM formation, which include medium type, oxygen tension, absence or

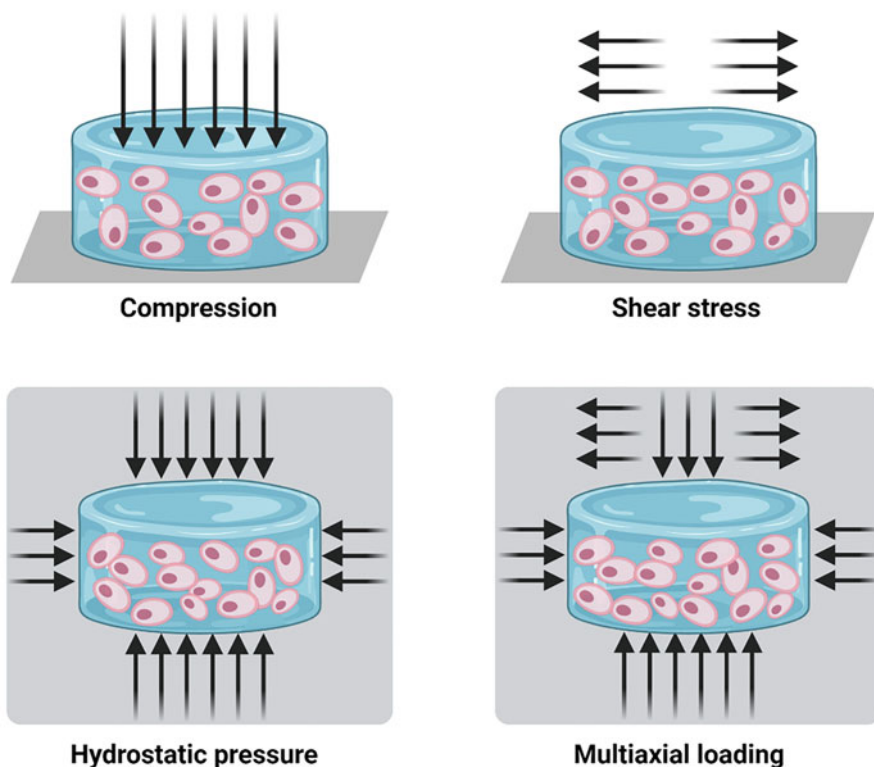


Fig. 5.1 Mechanical loading types. The commonly simulated types of mechanical stimulation in bioreactors are compression, shear stress, hydrostatic pressure (HP), and multiaxial pressure

presence of growth factors, and scaffold composition and structure. However, in this section we will focus on the influential effects of mechanical factors on cartilage formation in bioreactors.

5.4.1 Hydrostatic Pressure (HP)

In human hip joint, the stress sustained by cartilage is between 3 and 11 MPa (Afoke et al. 1987). Generally, the human walking cadence ranges from 1 to 2 Hz (Waters et al. 1988). These physiological magnitudes and frequencies are used as references for the design of HP-related bioreactors. There are two methods of exerting HP in bioreactors, namely by compressing gas phase or fluid phase (Elder and Athanasiou 2009). The latter is more widely used; it pressurizes the culture chamber via a piston controlled by a computer program (Fig. 5.2), without changing the gas concentration in the culture medium (Elder and Athanasiou 2009). To avoid changes of gas concentration and risks of contamination, Chen et al. designed a novel bioreactor that transmitted the HP to the culture medium via a flexible membrane in a

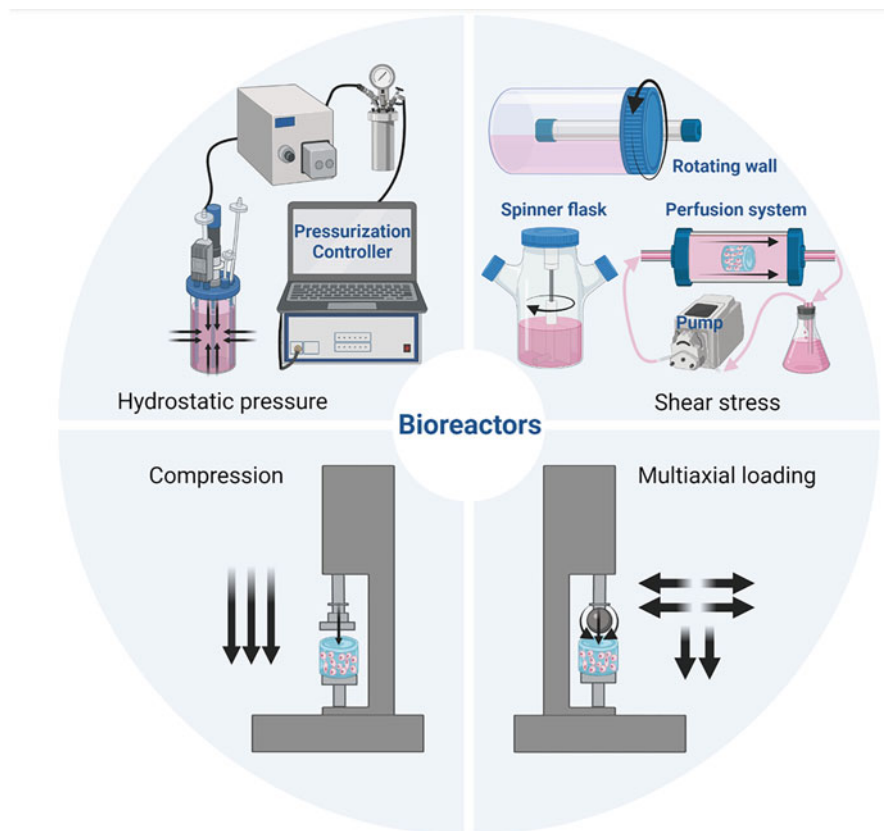


Fig. 5.2 Bioreactor types. Hydrostatic pressure-related bioreactors pressurize the culture chamber via a piston controlled by a computer program. To apply fluid shear stress, three kinds of bioreactors have been developed as shown in the figure. The compression bioreactors are widely used in scaffold constructs or explants. Different kinds of multiaxial loading bioreactors have been custom designed to better mimic native joint loadings

completely sealed chamber, which also had a large container space to culture multiple samples (Chen et al. 2017).

Both chondrocytes and stem cells have been influenced by HP bioreactors to facilitate cartilaginous tissue formation. The chondrocytes ranged from juvenile to mature cells, while the stem cells included adipose-derived MSCs (Correia et al. 2012), BMSCs (Luo et al. 2021), and embryonic stem cells (Luo et al. 2021). Additionally, suitable HP has been shown to enhance cartilaginous matrix synthesis in monolayer cells (Ikenoue et al. 2003), cartilage explants (Hall et al. 1991; Young et al. 2017), cell-laden scaffolds (Mizuno et al. 2002), and scaffold-free chondrocyte biomass (Kraft et al. 2011). Physiological loading (5 MPa) was more beneficial for chondrogenesis than low magnitude loading (0.4 MPa) in constructs of adipose-derived MSCs encapsulated in gellan gum hydrogels, as indicated by the results of

cartilage-related gene expression and histological staining (Correia et al. 2012). However, higher HP above physiological levels had limited or even detrimental effects on chondrocytes, explants, or tissue engineering constructs (Elder and Athanasiou 2009).

Dynamic HP has advantages over static HP. In a study by Correia et al. gene expression levels of aggrecan, collagen II, and SOX-9 in human nasal chondrocytes were higher in the group of pulsatile HP than steady HP over a period of 3 weeks (Correia et al. 2012). Similar results were observed in the study by Jortikka et al. in which 5 MPa dynamic HP increased GAG production, whereas 5 MPa static HP had no effect on GAG production (Jortikka et al. 2000). Multiple days of HP exposure are indispensable for a beneficial effect on cartilage formation. Luo et al. applied HP on ovine BMSC-laden scaffolds for 1, 4, 7, and 10 days, respectively, and found that increased GAG only existed in groups of 7 and 10 days of HP, and added collagen content only occurred in the group of 10 days HP, with shorter timepoints having no effects (Luo and Seedhom 2007). Similarly, Angele et al. demonstrated that human BMSCs loaded by HP for a single day had no changes in PG and collagen contents, whereas 7 days HP treatment resulted in significant increase in both (Angele et al. 2003). Aprile and colleagues found that the timing of HP application had bidirectional regulation effects on the cell fate of porcine BMSCs (Aprile and Kelly 2020). If applied at the onset of culture (from day 0 to day 7), HP inhibited chondrogenesis of MSCs and enhanced osteogenesis. In contrast, the delayed application of HP (from day 7 to day 14) could increase chondrogenesis of MSCs, cellular condensation, and aggregation. As for engineered cartilaginous tissue, the timing of HP exposure should be carefully taken into consideration due to its dual effects on stem cell fate.

There has been no consensus on the configuration of HP applied in bioreactors, which is mainly caused by numerous choices of HP parameters. However, even the same HP regime would lead to different responses in different *in vitro* culture systems. Divergent responses were observed in different cell sources (bovine/human cells), cell types (stem cells or chondrocytes), juvenile/adult chondrocytes, or even different cell passages (Elder and Athanasiou 2009). Additionally, different biomaterials for cell-seeded constructs may change the response of cells to HP stimulation. For each system, the optimal HP regime should be carefully considered, though physiological magnitude and frequency of HP are recommended by most studies.

5.4.2 Compression

During normal physiological movements, average compressive pressure in the knee and hip joint is approximately 0.5–7.7 MPa, and average deformation amplitude of cartilage can reach 13% of its total thickness (Mow and Wang 1999; Grad et al. 2011). Uniaxial compression is the most widely simulated type of load *in vitro*, owing to its relatively simple design in bioreactors. Likewise, the parameters of compression varied dramatically among different studies. Huang et al. demonstrated

that compared to TGF- β supplementation, cyclic compressive loading alone showed comparable chondrogenic ability in rabbit BMSCs agarose constructs (Huang et al. 2004). Though no consensus on compression load variables has been reached, many studies have shown that physiological compression magnitudes at 10% (Huang et al. 2004) or 15% (Campbell et al. 2006) led to upregulation of chondrogenic-related gene expression and GAG synthesis (Grad et al. 2011). After reviewing many studies, Anderson and Johnstone discovered that the optimal compression conditions, facilitating cartilaginous tissue formation in in vitro constructs, would be as following: dynamic compressive stimulation at physiological frequency of around 1 Hz; delayed load exposure after a preculture period; intermittent pressurization with substantial daily unloaded periods; and total loading duration more than 50 h (Anderson and Johnstone 2017).

5.4.3 Shear Stress

Normal physiological activity of diarthrodial joints gives rise to two kinds of shear stresses, namely fluid shear stress and contact shear stress (Sharifi and Gharraei 2019). Once exerting compression loading on cartilage during activity, the fluid in the tissue is expelled resulting in potential fluid shear stress at and around the cell membrane. When the pressure is discharged, the expelled water osmotically draws back and leads to fluid shear stress again (Smith et al. 1995; Lane Smith et al. 2000). Moreover, during activity articular cartilage surfaces rub against each other and thus produce contact shear stress. According to these mechanisms of shear stress formation, different bioreactors have been developed to explore the in vitro effects of shear stress on cartilage tissue engineering. Spinner flasks, rotating wall vessels, and perfusion bioreactors have been developed to impose fluid shear stress (Yeatts et al. 2013). These fluid shear bioreactors stimulate chondrocytes or constructs by the fluid flow in the chambers. As for the application of contact shear stress, various custom-made bioreactors have been introduced by different research groups. In these systems, additional compression pressure was typically superimposed simultaneously because contact shear stress usually accompanies compressive force during normal physiological situations.

5.4.4 Multiaxial Loading

During human gait processes, mechanical pressurization on articular cartilage is always a combination of HP, shear stress, and compression. To better mimic the native conditions, biaxial or even multiaxial loading systems have been developed (Wimmer et al. 2004). For example, Li et al. encapsulated human BMSCs in fibrin polyurethane scaffolds which were then imposed to various mechanical loading protocols of compression and shear stress (Li et al. 2010a). The study revealed that combined mechanical loadings enhanced chondrogenesis of human BMSCs, as indicated by upregulation of chondrogenic gene and protein expression. This effect

could be regulated by modifying mechanical frequencies and amplitudes (Li et al. 2010a). In addition, the combination of compression and shear was found to be essential for induction of BMSCs chondrogenesis, compared with compression or shear alone (Schatti et al. 2011). In tissue-engineered chondrocyte-seeded scaffolds, compression and shear load promoted the deposition of cartilaginous matrix within the construct and the formation of a layer of lubricin at the construct surface (Grad et al. 2012).

Apart from the abovementioned shear and compression biaxial bioreactors, other kinds of combinatory bioreactors have also been reported. Nazempour et al. investigated the effects of combined oscillating HP and shear stress on bovine chondrocyte-seeded agarose scaffolds in their single reaction chamber bioreactor system. In comparison with chondrocyte pellets after 21-day treatment, GAG and collagen secretions rose 1.6-fold with agarose encapsulation, 5.9-fold with 0.02 Pa shear stress, and 7.6-fold with synchronous 4 MPa oscillating HP (Nazempour et al. 2017). Besides their ability to promote anisotropy that is more similar to the native cartilage, multifactorial bioreactors are helpful to elucidate the roles of different mechanical loadings and interactions among them. Physiological stimuli in these systems may recreate cartilaginous tissue with better biochemical and biomechanical properties for cartilage regeneration.

5.5 Mechanotransduction Mechanisms in Chondrogenic Cells: Evidence from Bioreactors

Native joint loadings result in complex changes of strains around chondrocytes. Subsequent compression and shear stress cause cellular deformation (Guilak 1995), whereas HP has little effects on cell shape. Moreover, these loadings also lead to alteration of pericellular osmolarity and pH due to interstitial fluid flow (Guilak and Mow 2000). Though the specific pathways are not well elucidated, these biophysical parameters can further change the biochemical properties of chondrocytes and ECM. A number of *in vitro* studies have recapitulated the *in vivo* biophysical loadings with the application of bioreactors and discovered some invaluable findings. It is essential to further unravel the underlying mechanisms involved in the responses of chondrocytes or MSCs to the mechanical stimulation, in a bid to better deploy and design bioreactors to guide *in vitro* stem cell-based or chondrocyte-based cartilage tissue engineering. After reviewing the findings from *in vitro* studies with bioreactors, we classify the molecular mechanisms of the mechanotransduction process into four phases (Fig. 5.3).

In native articular cartilage, mechanical loadings stimulate chondrocyte metabolism and induce matrix synthesis to maintain its homeostasis. Mechanical stimuli result in immediate biophysical and biomechanical changes like cell deformation, hydrostatic pressure, osmotic pressure, fluid flow, and release of local molecules in the pericellular milieu (Zhao et al. 2020; Ramage et al. 2009). These alternations are then sensed by mechanoreceptors on the chondrocyte surface, triggering intracellular signaling pathways (Ramage et al. 2009).

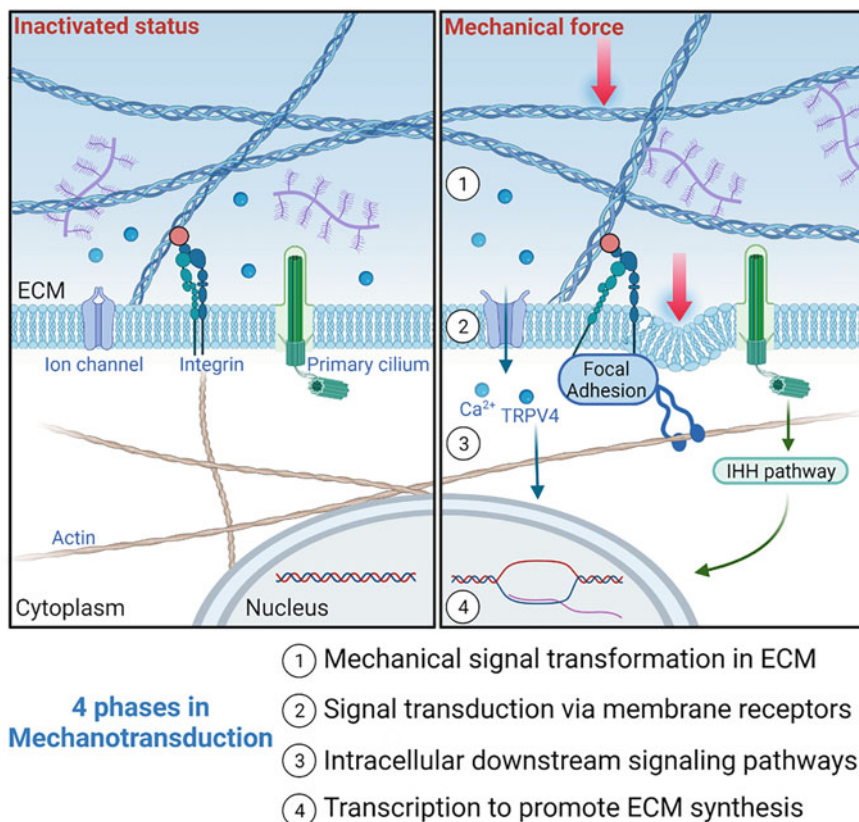


Fig. 5.3 Mechanical signal transduction mechanisms with evidence from studies on bioreactors. The process can be divided into 4 phases. Once applying mechanical forces, the biophysical and biochemical milieu in the ECM changes immediately, which can then activate the membrane receptors. The activated mechanoreceptors trigger intracellular downstream cascade signaling pathways, resulting in transcription factors translocating into the nucleus to promote chondrogenic-related gene expressions

5.5.1 Mechanoreceptors on Chondrocyte Cytoplasmic Membranes

5.5.1.1 Ion Channels

In chondrocytes ion channels include sodium (Na^+) channels, potassium (K^+) channels, calcium (Ca^{2+}) channels, and chloride (Cl^-) channels (Barrett-Jolley et al. 2010). Different forms of mechanical stimuli can alter the pericellular osmolarity which further regulates various ion channels in chondrocytes (O'Connor et al. 2013). HP could inhibit Na^+/K^+ cation pump and $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransporter in bovine chondrocytes, though their response varied depending on pressure amplitude, duration, and intracellular Na^+ concentration (Hall 1999). Browning et al. found a significant intracellular Ca^{2+} increase in bovine articular chondrocytes under exposure to high HP by mobilizing intracellular Ca^{2+} pools (Browning et al. 2004).

Moreover, intracellular Na^+ was also affected by a rise in osmotic pressure and HP (Browning et al. 2004). Another study showed that fluid shear could also upregulate intracellular Ca^{2+} concentration (Yellowley et al. 1999). A later study by Han et al. applied compressive loads to femoral condyle cartilage of rabbits and observed an instantaneous Ca^{2+} influx, in contrast to delayed Ca^{2+} signals in isolated chondrocytes (Han et al. 2012). Likewise, in in situ calf cartilage explants, Lv et al. found that extracellular Ca^{2+} was indispensable in intracellular Ca^{2+} responses to compression stimulation (Lv et al. 2018). Under compressive loading, inhibitors of K^+ and Ca^{2+} channels affected GAG content, suggesting ions played a role in transduction of mechanical stimulation and GAG synthesis (Mouw et al. 2007). Transient receptor potential vanilloid 4 (TRPV4), a specific Ca^{2+} channel, acted as a transducer of mechanical loading to regulate cartilage ECM biosynthesis, which could be targeted to enhance matrix formation and mechanical properties in tissue engineering constructs (O'Connor et al. 2014). Different kinds of loadings with various parameters could alter ion transporter systems in chondrocyte cytoplasmic membranes, which would further influence intracellular ion concentrations, regulate cell metabolism, and affect cartilage formation.

5.5.1.2 Primary Cilia

Primary cilium is a long organelle, protruding from the cell surface of nearly all cell types into the pericellular microenvironment. It also exists in chondrocytes as a versatile regulator of cartilage homeostasis and development, including matrix secretion, and mechanically mediated chondrogenesis (Tao et al. 2020; Wheatley and Bowser 2000; Poole et al. 1985). Wann et al. found that primary cilia were indispensable for compression-induced signaling transduction, upregulation of aggrecan gene expression, and GAG secretion in chondrocyte-laden agarose constructs (Wann et al. 2012). In both bovine- and human-derived tissue-engineered cartilage, fluid-induced shear stress improved neocartilage mechanical properties, by activating the mechanically gated complex on chondrocyte primary cilia (Salinas et al. 2020). A study from Shao et al. revealed that in rat growth plate chondrocytes, primary cilia increased IHH signaling expression under hydrostatic loading (Shao et al. 2012). Biological and chemical removal of primary cilia abolished upregulated gene expression levels of collagen II (Col II), collagen X (Col X), and bone morphogenetic protein 2 (BMP-2) under 10% cyclic loading in mouse chondrogenitor ATDC5 cells (Deren et al. 2016).

5.5.1.3 Integrins

Integrins are transmembrane heterodimeric proteins consisting of α and β subunits, which connect the pericellular matrix (PCM) to the intracellular cytoskeleton in cartilage (Dieterle et al. 2021). Integrins help to maintain cell shape and transmit physical loadings from the ECM to the cytoskeleton via focal adhesion (FA) proteins, like FA kinase (FAK) and tyrosine kinase Src (Zhang et al. 2018). Moreover, FA proteins and cytoskeleton influence MSC differentiation fate (Mathieu and Lobo 2012). Blocking integrins on chondrocytes encapsulated in porous constructs by antibodies inhibited cell spreading and reduced compression-

induced increase in matrix accumulation (Spiteri et al. 2010). In a similar study, Kock et al. blocked integrin receptors by GRGDSP, which counteracted the upregulated ECM synthesis by compressive loading in chondrocyte-seeded agarose constructs (Kock et al. 2009). Steward et al. revealed that under mechanical loading, matrix stiffness modulated PCM, integrin, and cytoskeletal structure and influenced cartilage formation of MSC-seeded constructs (Steward et al. 2013, 2014).

5.5.2 Downstream Signaling Cascades

Once detected by mechanoreceptors, extracellular physical signals subsequently transform into intracellular biochemical signals and trigger downstream signaling networks. Numerous studies have revealed involvement of many signaling pathways in mechanotransduction, like TGF- β , Wnt, and MAPK pathways (Zhao et al. 2020). Moreover, integrins and FA proteins transduced physical signals mainly via RhoA/ROCK and Hippo/YAP/TAZ pathways (Zhang et al. 2018). IHH signaling pathway was increased under hydrostatic loading stimulation, which was modulated by primary cilia (Shao et al. 2012).

Usually, in one experimental study only one signaling pathway was proved to be involved in mechanotransduction under specific bioreactor conditions. Nevertheless, some studies revealed that more than one signaling transduction pathway was related to mechanical stimulation, which should better reflect the authentic mechanotransduction situation in native cartilage. In the absence of any growth factor, compression and shear force have shown to stimulate chondrogenesis of BMSCs by enhancing the endogenous expression of TGF- β 1 and TGF- β 3 at both gene and protein levels (Li et al. 2010b). Raizman et al. showed that both integrin- and calcium-mediated pathways responded to compression stimulation and converged on extracellular signal-regulated kinase (ERK) in mitogen-activated protein kinase (MAPK) pathway in bovine chondrocyte-seeded tissue engineering constructs (Raizman et al. 2010). In regulation of human MSC chondrogenesis and hypertrophy under dynamic compression, the crosstalk between mechanoreceptor integrin β 1 pathway and classical TGF- β /SMAD chondrogenic pathway plays an important role for the cell differentiation fate (Zhang et al. 2015). Downstream effectors of the abovementioned signaling pathways translocate into the cell nucleus and modulate chondrogenic gene expression, promoting cartilage matrix formation or degrading its matrix. However, the mechanism bridging these pathways to chondrogenic protein accumulation and neocartilage formation remains an unsolved question for further exploration.

The parameters related to the application of bioreactors vary dramatically among studies and need to be adjusted for each culture system to achieve optimal output for cartilage tissue engineering. In addition, it is also important to utilize bioreactors to dig into undiscovered mechanisms, novel biomarkers, or signaling pathways, to elucidate how extracellular mechanical loadings are transduced into intracellular organelles and then influence cell physiological functions. The future in vitro

cartilage engineering strategies and regenerative therapies may benefit from these innovative findings.

5.6 New Perspectives for Mechanically Stimulated Cartilage Models: Joint-On-Chip

The *in vitro* studies that intend to test drugs for traumatic or degenerative joint diseases or assess the behavior of cells and tissues require reproducible models that can produce stable results. For decades, one of the promising fields under investigation has focused on using microfluidic systems and culture platforms called organ-on-chip (OOC) to mimic the key organotypic cellular architecture and functionality (Huh et al. 2013). This technique intends to combine the culture of different cell types with surrounding conditions like ECM, nutrients, drugs, and mechanical stimuli in one solution (Fig. 5.4). Unlike tissue engineering, the organ-on-chip culture techniques do not have the primary purpose of creating an entire tissue that can subsequently be implanted for a clinical approach to restore the lost or damaged structure; instead, the main objective is to study the behavior of cells cultured in a microenvironment that represents the most physiological condition possible (Wang

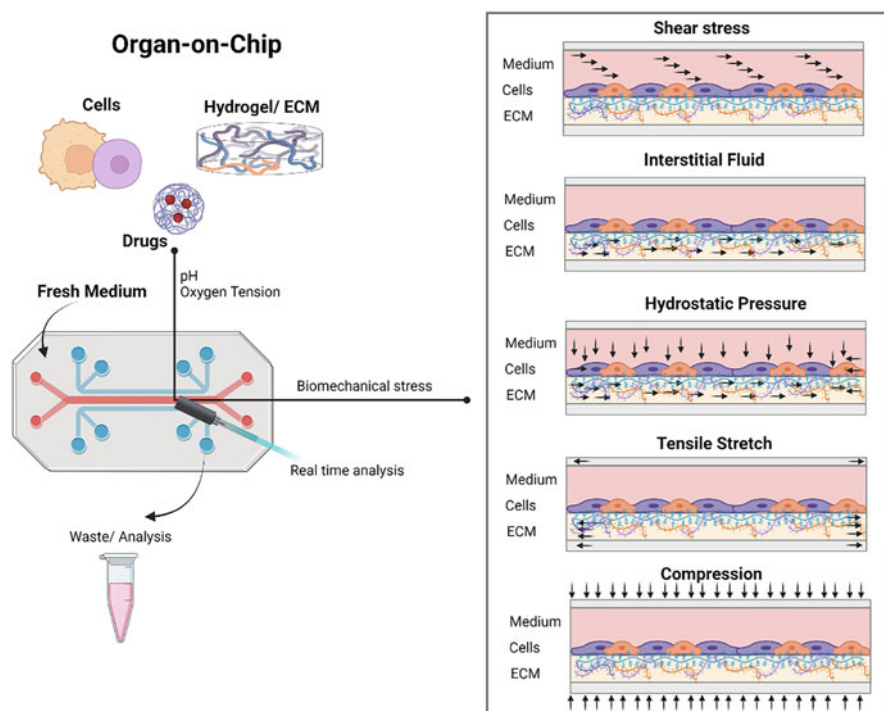


Fig. 5.4 Future prospect of cartilage formation: cartilage-on-chip

et al. 2016). The main purposes are the possibility of replacing and reducing the use of animal studies, and allowing the assessment of a pharmacological response in a specific patient, intending to apply a precision-based medicine (Murphy and Atala 2016). The conventional approach implies the culture of a single cell type or multiple cells in a 2D or 3D system. There are many organ-on-chip companies on the market. However, the products are similar; currently, the most common OOC tools are based on organic polydimethylsiloxane (PDMS) fabricated by the standard soft lithography technique that supports the growth of cells on specific extracellular matrices. The chips are compact and can be organized in several chambers. In each, all nutrients, drugs, flow rate, pressure, oxygen tension, and pH are controlled by external devices like a peristaltic pump or sensors (Huh et al. 2013). The new chips allow the setting of the mechanical stimuli over time in a different controlled manner. The biomechanical stimulation, indeed, is of fundamental importance for the physiological regulation and organization of many types of tissue (Thompson et al. 2020). Cells and tissues, *in vivo*, are subjected to biomechanical stimuli, which can be passive or active. Passive stimuli include stiffness, geometric confinement, or topographic cues. Active stimuli include connective tissue tensile stretch and compression, fluid shear stress, interstitial fluid flow, and hydrostatic pressure (Thompson et al. 2020).

There are numerous commercial models in the musculoskeletal and orthopedic field, specifically for bone, cartilage, and tendon. The main OOCs permit to apply stimuli such as fluid shear stress, hydrostatic pressure, and tensile stretch (Fig. 5.4). For example, the Emulate system has recently been used for the study of bone differentiation from BMSCs in the presence of BMP2 at a flow of 30 $\mu\text{L}/\text{h}$ with a shear stress of 0.346 MPa, indicating an increase in cell viability and expression of bone molecular markers (Sheyn et al. 2019). In another model of organ-on-chip, the TissUse OOC, BMSCs were cultured on a scaffold of hydroxyapatite and zirconia (Sieber et al. 2018) under a flow rate of 5 $\mu\text{L}/\text{min}$ at a frequency of 2 Hz for 28 days, demonstrating the possibility of conducting hybrid studies of tissue engineering and physiology in a controlled manner. Furthermore, this type of technology would not only allow studying the cellular response to drugs in a more physiological state in healthy and pathological models of tissues but could give the possibility to monitor the cellular response in real time with non-destructive techniques, thus limiting costs and time. Usually, this type of technology has a fluidic system that permits the dynamic change of medium, efficiently promoting the study of the analytes produced over time. Recently, new systems have been developed that involve sensors for metabolites, pH, and salts, allowing direct monitoring of these parameters (Kieninger et al. 2014).

Currently, the OOC studies are mainly present in academic laboratories and less in pharmaceutical industries, although their use would limit time and costs. Although organ-on-chips have been studied for several years, from a technical point of view, there are still problems limiting the standardization and use on the extended and optimized level (Low and Tagle 2017). For example, the formation of bubbles in the fluidic system can easily undermine the standardization of results. Furthermore, it is currently possible to run only a limited number of samples at a time; in scenarios

such as the pharmaceutical industry, this represents a huge limitation that affects automation and high throughput. The use of OOC in clinical practice and for personalized medicine would be of considerable interest. However, unfortunately, the major problem is still the standardization of the process and possible analysis in real time. Nevertheless, the OOC technology is a potential route toward the drug development pipeline, which currently suffers substantial and costly attrition. To date, there is already significant interest and investment in this area from the biotech and pharmaceutical industries. Indeed, it is estimated that OOC technology could reduce pharmaceutical research and development costs by 10–26% (Franzen et al. 2019).

Cartilage-on-a-chip models have been developed to mimic diseases and enable the screening of new drugs and therapeutics. In a three-dimensional cartilage microtissue model, compressive load was applied in a strain-controlled manner (Occhetta et al. 2019). Hyper-physiological compression of 30% strain could induce phenotypic changes that were consistent with catabolic, pro-inflammatory, and hypertrophic responses in osteoarthritic chondrocytes. The model was successfully validated using standard therapies such as steroids and non-steroidal anti-inflammatory drugs. While the cartilage-on-chip culture system only consists of one cell type, namely chondrocytes, more complex joint-on-chip models and mini organ cultures have also been described. Currently existing systems and future developments were comprehensively reviewed in a recent article by Paggi et al. (2022). The joint-on-a-chip aims to recapitulate the interaction of the different cell types within an articular joint, including articular chondrocytes, fibro-chondrocytes, bone cells (osteoblasts, osteoclasts), synovial cells, immune cells, endothelial cells, adipose tissue-derived cells, and neural cells. In these mini organs, the diverse mechanical stresses that are experienced by the various cells and tissues can be simulated by perfusion and compression systems. The mechanical stimulation is typically provided by microfluidics as described above or by mini-bioreactor devices (Nichols et al. 2018). Nevertheless, despite recent progress, the complex manufacturing and challenging cell and tissue maintenance limit the standardization and throughput of such engineered miniaturized systems. Thus, the practicality of each type of bioreactor for culture of cells, tissues, or whole organs strongly depends on the intended use. While complex systems may be suitable for fundamental studies, highly reproducible and well-standardized techniques are likely more appropriate for high throughput screening purposes.

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Signaling Pathways Regulating Cartilage Formation

6

Faiza Ramzan, Asmat Salim, and Irfan Khan

Abstract

Over the course of many years of investigation, the molecular processes that regulate the differentiation of chondrocytes throughout the development of cartilage from their initial activation from mesenchymal progenitor cells to their eventual maturation into hypertrophic chondrocytes have been discovered. In this chapter, we take a glance at the interaction between a number of signaling molecules, mechanical cues, and morphological cell characteristics to activate a specific subset of crucial transcription factors that regulate the genetic program that triggers chondrogenesis and chondrocyte divergence, which leads to the formation of cartilage. We also discuss current research on how various signal transduction pathways regulate chondrocyte differentiation and multiplication in the articular surface. In adult normal cartilage, the anabolic and catabolic processes of chondrocyte maturation are delicately balanced. Due to the degradation of joint with age, the body's ability to maintain homeostasis is compromised, catabolic pathways are triggered, and cartilage is acutely and severely prone to degeneration. Because the differentiation of cartilage and maintenance of cellular metabolism are intricately governed by a complex series of signal transduction and biophysical elements of the system, it appears that recognizing these processes will be beneficial for both exploring the molecular and biological methods for cartilage tissue engineering and identification of the disease-causing major elements for particular therapeutics for management of the disease progression. This chapter will emphasize on the key signaling pathways that can activate the cellular, subcellular, and biochemical mechanisms, controlling functional

F. Ramzan · A. Salim · I. Khan (✉)

Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan

e-mail: khan@iccs.edu

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M. Baghaban Eslaminejad, S. Hosseini (eds.), *Cartilage: From Biology to Biofabrication*, https://doi.org/10.1007/978-981-99-2452-3_6

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properties of the cartilage under normal circumstances. These pathways may have an impact on how various cartilage tissue compartments interact. Consequently, the study in this area may result in the development of more efficient cartilage regeneration therapies.

Keywords

Cartilage signaling pathways · Chondrocytes · BMP · GDF-5 · IGF-1 · FGF · Hedgehog signaling · Notch signaling

6.1 Introduction

Osteoarthritis, the most severe type of arthritis, is defined as the degeneration of bone and cartilage. Alteration in the mechanical properties of articular cartilage and chondrocyte metabolic activity, both of which are closely correlated to aging or injury, leads to degradation of the matrix, which results in severe pain and disability (Shah et al. 2007). Because the articular cartilage has limited ability to regenerate on its own, both surgical and non-surgical treatments have been used to heal the tissue and reduce pain (Smith et al. 2005). However, providing their short-term effectiveness, current therapy options are insufficient to alter the diseased condition. Despite its prevalence, there are still few effective treatment choices for osteoarthritis (Wieland et al. 2005). Cartilage is an avascularized and highly specialized tissue made up primarily of chondroitin sulfate, aggrecan, and collagen, mainly type II, IX, and XI in the form of an extensive extracellular matrix (ECM) (Elder et al. 2009). This highly ordered fibrillar architecture contributes to the tissue architecture, stiffness, tensile strength, and compressive resistance, providing cartilage biochemical properties (Wieland et al. 2005). Chondrocytes present in the cartilage regulate the formation, stabilization, and destruction of ECM proteins. In healthy adult cartilage, the cells are in a resting state, which is distinguished by a delicate balance between processes of biosynthesis and degradation (Goldring and Marcu 2009; Poole et al. 2001). With age, degenerative joint diseases, and traumatic cartilage lesions, physiological settings are lost and catabolic pathways are upregulated (Wang et al. 2011a). The most prevalent chronic joint disease, osteoarthritis (OA), is known to be caused by a variety of major drivers or transcription regulators for progressive cartilage degradation, which include chemokines, ECM-degrading enzymes like aggrecanase, disintegrin, and metalloproteinase with thrombospondin motifs, as well as pro-inflammatory cytokines (IL-1, TNF). Changes in chondrocyte differentiation are another indicator of cartilage degeneration (Fosang and Beier 2011).

Furthermore, to expressing markers of terminal differentiation such as Runt-related transcription factor 2 (RUNX-2), collagen X, MMP-13, and Indian hedgehog (Ihh), hypertrophic chondrocytes also develop an “autolytic” phenotype, which is indicated by their ability to result in the breakdown of intercellular cartilage matrix (Rasheed et al. 2011; Minina et al. 2002; Caplan et al. 1997; Hunziker 2002;

Johnstone et al. 2012; Decker et al. 2017). In contrast, Wnt repressors, like Dickkopf (Dkk-1), are Wnt signaling-blocking systems that defend cartilage and suppress the expression of genes implicated in hypertrophy (Archer et al. 1995). The interplay of the FGF, TGF- β , BMP, and Wnt signaling pathways, which decide whether they reside within cartilage or pass through hypertrophic maturation before ossification, is another complex regulatory mechanism that governs chondrocyte fate (Rasheed et al. 2011). In particular, it has been shown that FGF signaling accelerates terminal hypertrophic differentiation, while BMPs slow it down. Therefore, FGF signals block BMP and reduce *Ihh* transcription. Cartilage has limited self-regeneration capacity. Damage brought on by aging, injury, and pathological experiences causes failure in tissue repair processes, resulting in a newly formed tissue that is malfunctioned, causing constrained capacity of cartilage for self-regeneration (Minina et al. 2002; Caplan et al. 1997; Hunziker 2002). Therefore, due to the absence of self-regeneration, a number of procedures, including surgical methods, transplantation methods, tissue engineering techniques, and bio-regeneration technologies, have been used for the management of cartilage lesions (Johnstone et al. 2012). Understanding the signaling cascade is essential for both the formulation of biological strategies for enhancing cartilage regeneration and the selection of key molecular targets for the treatment of cartilage defects.

The critical processes that underlie cartilage physiology are the focus of this chapter. We focus on the crucial signals, such as transforming growth factor beta (TGF- β), bone morphogenetic proteins (BMPs), insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF), and Wnt/-catenin, growth differentiation factor (GDF-5), hedgehog (Hh), and notch pathways that can activate the key transcription modulators for chondrocyte maturation, proliferation, and successively lead to cartilage formation and regeneration (Fig. 6.1).

6.2 Development and Organization of the Cartilage

A specialized tissue called articular cartilage anatomically plugs epiphyses at its ends in the synovial joint cavity. Adult articular cartilage exhibits constituents including type II collagen, proteoglycans, and sulfated glycosaminoglycan (Decker et al. 2017). In particular, articular cartilage lacks cells, nerves, lymphatic vessels, and blood vessels with minimal to no cell turnover. Chondrocytes in the articular cartilage help maintain the extracellular matrix microenvironment (Archer et al. 1995; Sophia Fox et al. 2009). In extracellular matrix of the cartilage, longitudinally oriented type II collagen and GAGs, such as chondroitin sulfate and hyaluronic acid, are linked to one another via core proteins (Sophia Fox et al. 2009). These protein complexes also contribute to the formation of complex network of extracellular matrix that absorbs the mechanical pressures applied on the articular cartilage (Carter et al. 2004). The lubricated smooth surface of articular cartilage, which is another structural characteristic and is made up of lubricin and collagen fibers that are horizontally aligned, reduces friction caused by skeletal motion (Camarero-Espinosa et al. 2016; Hughes et al. 2005). Articular cartilage is made up of several

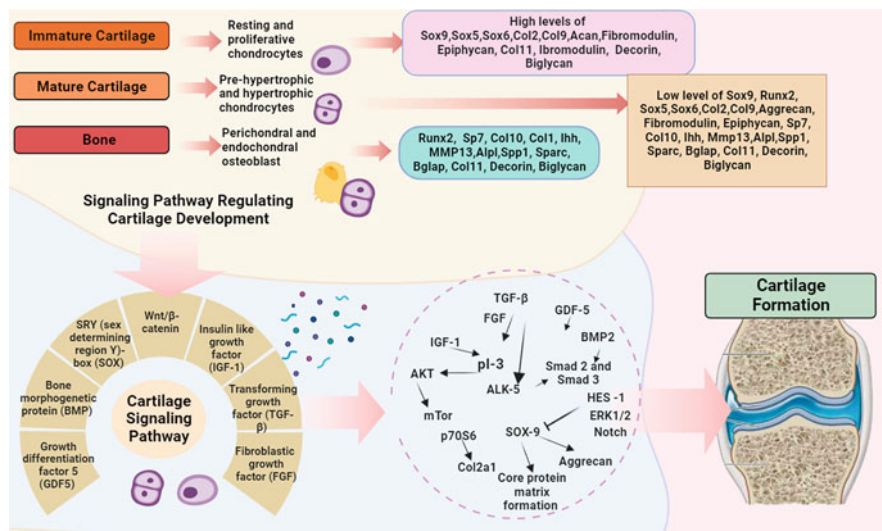


Fig. 6.1 Stages of the development and the signaling pathways regulating cartilage formation: Chondrocytes in immature, mature cartilage, and the bone are under the control of SOX-9 and RUNX-2 during endochondral ossifications. The upregulation of different genes is under the influence of SOX-9 or RUNX-2. When endochondral ossification occurs, osteoblasts in the perichondrial and endochondral bones express RUNX2 gene, while chondrocytes in mature cartilage only moderately express SOX-9 genes. In each of these three different subtypes of skeletal cells, COL-11, Decorin, and Biglycan expression is evident. Immature cartilage, adult cartilage expressed different signaling cascade that regulates and develops the articular surface. However, the interaction of multiple signaling cascade leads to the formation of entire cartilage tissue

layers with orientated cell populations and fiber matrices. The majority of the skeletal structure in vertebrate develops from cartilage through a natural biological process called endochondral ossification (Kozhemyakina et al. 2015). Mesenchymal condensation at the potential site of bone results in the formation of cartilage anlagen in the first stage. As chondrocytes proliferate and differentiate, cartilage anlagen develops a cartilage template that resembles the morphology of future bones. Apoptosis followed by hypertrophic differentiation and chondrocyte maturation occurs in the cartilage center, causing vascular invasion and osteoblast ossification (Long and Ornitz 2013). This subsequent occurrence spreads to the metaphysis longitudinally. Later, the epiphysis develops a second ossification site that expands radially within it. This site is known as the secondary ossification center (Lefebvre and Bhattaram 2010). A segment of cartilage between the two ossification centers maintains a growth plate or physis during skeletal development, whereas other segments between the joint space and the secondary ossification center develop into articular cartilage and remain there for life.

6.3 Signaling Pathways Regulate Chondrogenesis During Cartilage Formation

Numerous distinct signaling pathways are involved in the formation of cartilage or chondrogenesis. The mechanism for cartilage development is initiated when mesenchymal stem cells (MSCs) migrate from the lateral plate mesoderm to the limb field region, where they condense through a process named as condensation (DeLise et al. 2000). For chondrocyte differentiation, the condensation process which is controlled by interactions between the cells and matrix is essential (Caplan et al. 1997). The formation of cartilage anlagen results from the differentiation of stem cells into chondrocytes, which accumulates ECM, including proteoglycans and collagen types II, IX, and XI (Hunziker 2002). During the process of endochondral ossification, the middle chondrocytes in the condensation zone develop into hypertrophic cells that produce type X collagen (DeLise et al. 2000; Demoor et al. 2014). Next comes the commencement of the interzone and the development of the epiphyseal ossification center, which defines the developing joint (DeLise et al. 2000; Demoor et al. 2014). The articular cartilage is then formed by the cells near the border of cartilage anlagen (Caplan et al. 1997). The processes of chondrogenesis and hypertrophy are controlled by a variety of signals. Growth factors and transcription factors can influence the chondrocytic phenotype or promote the synthesis of cartilage matrix and help maintain the homeostasis and regeneration (Fig. 6.2).

6.4 Early-Stage Regulator in the Signaling Cascade

The growth factors that constitute the TGF- β superfamily, including TGF- β s, BMPs, Sox-9 Wnt/catenin, and growth differentiation factors (GDFs), engage with receptors to initiate the signaling pathways necessary for the growth of tissue and maintenance of homeostasis (Umlauf et al. 2010). They are largely expressed in chondrocytes and are crucial for maintaining the formation and regeneration of cartilage tissue (Umlauf et al. 2010).

6.4.1 TGF- β Signaling Pathway

TGF- β , a group of representative proteins, is thought to have an impact on cartilage development. TGF- β signals are delivered via type II receptors, which also draw and phosphorylate type I receptors, activating SMAD proteins, SMAD2 and SMAD3 and the targeted gene expression occurs when co-Smad (Smad4) and phosphorylated Smad proteins interact, which initiate the cascade (Vautier et al. 2010). Smad3 rather than Smad2 mediates the majority of TGF- β signaling during chondrogenesis and chondrocyte maturation (Ferguson 2000; Furumatsu et al. 2005). To control chondrogenesis, TGF- β may also activate p38, MAP kinase pathways, as well as extracellular signal-regulated kinase (ERK) (Li et al. 2010). TGF- β isoforms such as TGF- β 1, β 2, and β 3 are present in the hypertrophic cartilage in the growth

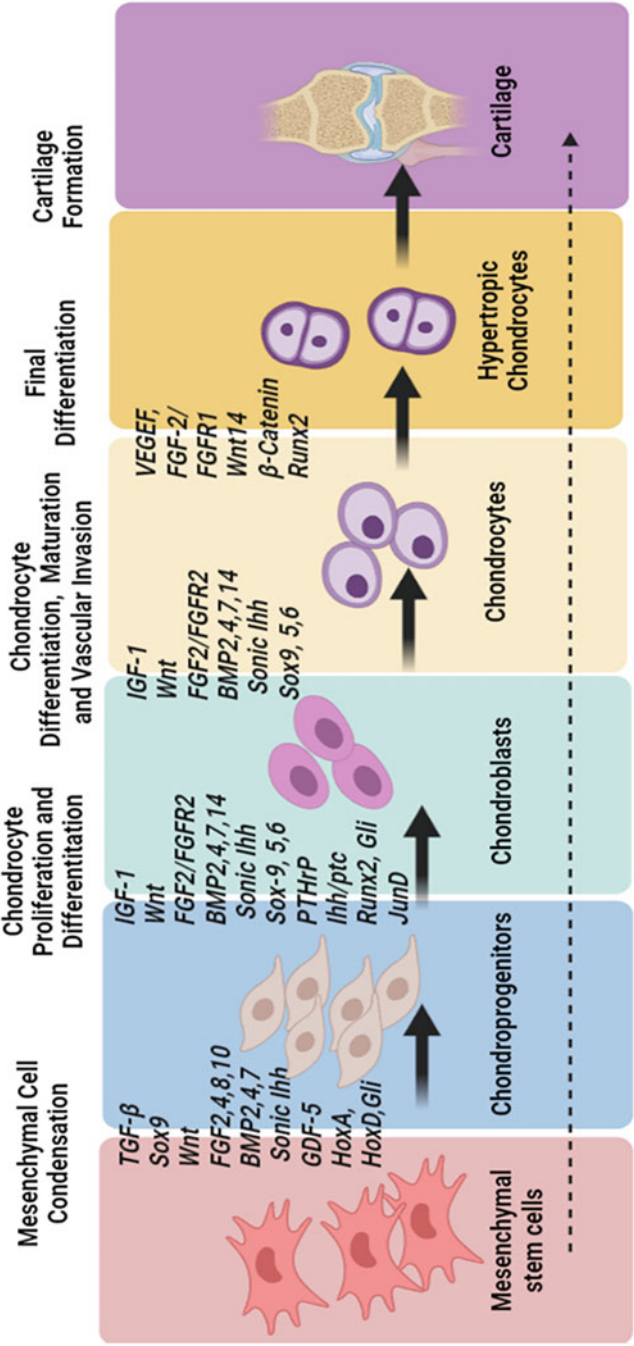


Fig. 6.2 Signaling molecules regulate the development of cartilage: Regulatory molecules and the gene transcriptional factors involved in the mechanism of cartilage development

plate as well as in the periosteum and perichondrium. The continued expression of TGF- β 1 and TGF- β 2 in mature articular cartilage suggests that these proteins are important for both the development and maintenance of articular cartilage (Pogue and Lyons 2006). TGF- β s have a significant impact on all stages of chondrogenesis, including condensation of MSCs, chondrocyte maturation, proliferation, ECM production, and hypertrophic chondrocyte development (Pogue and Lyons 2006). MSC adherence promotes cell interactions in the early stages of condensation. The ECM proteins that the cells stick to, such as fibronectin and tenascin, as well as adhesion molecules like N-cadherin and N-CAM, are affected TGF- β 1, β 2, and β 3 (Oberlender and Tuan 1994). Wingless-Int (Wnt) signaling, c-Jun N-terminal kinase (JNK), MAP kinases, ERK, p38, as well as TGF- β -mediated N-cadherin expression regulation are all involved in mesenchymal progenitor cell synthesis (Long and Ornitz 2013; Widelitz et al. 1993; Frenz et al. 1989). In addition to its function in condensation of MSCs, TGF- β promotes cell proliferation and the production of glycosaminoglycans (GAGs), a component of cartilage matrix, as well as the expression of genes unique to cartilage, like aggrecan and type II collagen (Darling and Athanasiou 2005). It has been found that SOX-9-mediated transcription is stimulated by TGF- β -activated Smad3/4. This happens when SOX-9 attaches to the enhancer region of type II collagen gene and interacts with active Smad3 and p300, a transcriptional coactivator (Khalid et al. 2022; Kulyk et al. 1989). TGF- β s serve as chondrogenesis stimulators, and their interactions with other signaling molecules initiate the process. In the initial phases of chondrogenesis, TGF- β s work as stimulators; however, as chondrocyte differentiation progresses, they act as inhibitors. By preventing the transcription of collagen X, MMP-13, VEGF, and osteocalcin, TGF- β prevents the formation of hypertrophic chondrocytes (Furumatsu et al. 2009). TGF- β inhibitory action is carried out through Smad2/3 signaling, which is crucial for preventing hypertrophy from developing further (Ferguson 2000). In a research using mutant mice with a targeted deletion of Smad3, an abnormally large number of hypertrophic chondrocytes were found, indicating the critical role of Smad signaling in chondroblast TGF- β -induced terminal differentiation. Furthermore, Runt-related transcription factor-2 (RUNX-2), a key transcription involved in osteoblast development and chondrocyte maturation (Pogue and Lyons 2006), engages with Smad3 induced by TGF- β to inhibit RUNX-2 function (Oberlender and Tuan 1994). The TGF- β signaling cascade's simultaneous role in regulating chondrogenesis and hypertrophic maturation, as well as the concentrations of these proteins at particular developmental stages, may have a significant impact on cartilage development (Oberlender and Tuan 1994) (Fig. 6.3).

6.4.2 BMP Signaling Pathway

In different phases of skeletal growth, BMPs are considered as one of the crucial signaling factors for the development of bone and cartilage. Since they promote mesenchymal cell commitment to the chondrocytic lineage, cell proliferation, and chondrocyte hypertrophic differentiation in the epiphyseal plate and have a critical

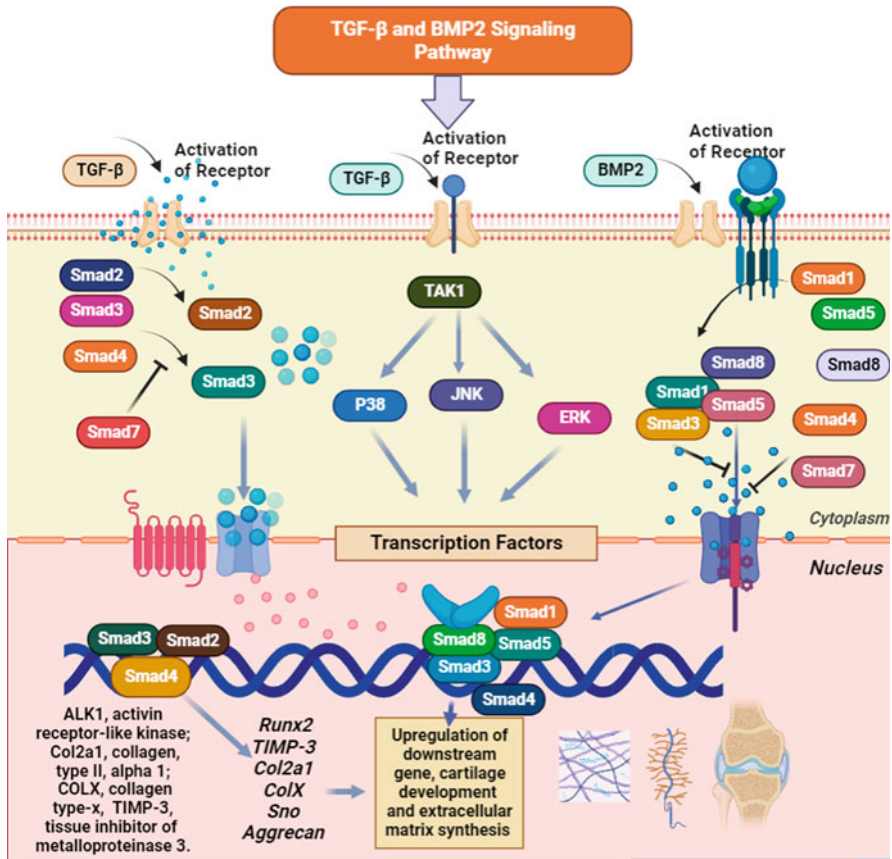


Fig. 6.3 The classical Smad-dependent cascade of TGF-β2 and BMP2 governs the development of cartilage: The conventional Smad-dependent signaling pathway is activated when active TGF-β2 binds to its receptors. TGF-β2 signals also have an impact on BMP signal transduction. This initiates the Smad-dependent downstream signaling cascade, which regulates the chondrocyte maturation and the extracellular matrix production which lead to cartilage development

impact on the formation of cartilage and bone *in vivo*, they are termed as crucial signaling factors for chondrogenesis and osteogenesis (Pogue and Lyons 2006). Despite the fact that both TGF-βs and BMPs belong to the TGF-β superfamily, their effects on chondrogenesis are regulated by completely different signaling pathways (Vautier et al. 2010). The P38 and MAP kinase signaling cascade has been linked to BMP-induced chondrogenesis (Liao et al. 2014).

BMP signaling plays a function in chondrogenesis through the mediation of the SOX family. Three members of the SOX family SOX-9, L-SOX-5, and SOX-6 have received a great deal of attention as transcriptional regulators of chondrogenesis. When SOX-9 is expressed, BMP signaling is downregulated and cartilage marker expression is stimulated (Zehentner et al. 1999). It has been demonstrated that BMP

stimulates SOX-6 in a manner that is similar to BMP and is time-dependent. After BMP stimulation, there was an increase in SOX-6 binding to the promoter of type II collagen gene, which indicates that SOX-6 is essential for modifying BMP signaling in chondrogenesis (Darling and Athanasiou 2005). Furthermore, a study showed that SOX-9, L-SOX-5, and SOX-6 expression was suppressed during condensation in mice with dual mutations of BMP receptor type 1A and B (BMPR1A and 1B), where BMPs bind to transmit their signals, implying the significance of BMP signaling for maintaining SOX protein synthesis (Yoon et al. 2005) (Fig. 6.3).

In *ex vivo* limb culture, overexpression of SOX-9 was found to enhance BMP-induced chondrogenic differentiation and has synergistic effect with BMP on chondrocyte condensation and proliferation (Liao et al. 2014). An important regulation of chondrogenesis is the interaction between BMP signaling and SOX expression. In addition of their roles in the initial stages of chondrogenesis, BMPs also show their participation in later growth plate phases by promoting chondrogenic multiplication and enlargement (Pogue and Lyons 2006). When noggin, a BMP signaling antagonist, was added, the effects of the administration of BMP, which promoted longitudinal progression of the metatarsal bone and stimulated chondrocyte proliferation and hypertrophy in the growth plate, were blocked (De Luca et al. 2001). The hypertrophic zone was larger in transgenic mice that expressed BMP under the direction of the collagen XI gene promoter/enhancer; this was presumably caused by the accelerated hypertrophic differentiation of chondrocytes (Leboy et al. 2001). Immature chondrocytes, rather than fully developed hypertrophic chondrocytes, were discovered in noggin over-expressing mice (Leboy et al. 2001). Due to noggin expression during the development of growth plate cartilage, it is possible that the concentration and action of BMP are regulated by the activities of its antagonist. BMP-2, 4, 7, and GDF-5, as well as other BMP ligands, have all been shown to have roles in the growth plate formation and chondrogenesis. The MSC condensation process requires BMP-2, which also promotes the formation of cartilage matrix proteins (Liao et al. 2014). By activating type X collagen, it also causes the hypertrophic differentiation of proliferating chondrocytes in the growth plate. It has been demonstrated that type X collagen gene transcription is promoted by BMP-2-stimulated Smad1/5 in conjunction with RUNX-2 to control chondrocyte hypertrophy (Liao et al. 2014). Similarly, by enhancing the expression of type II collagen and aggrecan, BMP-4 encourages the development of cartilage matrix.

On the other hand, type X collagen expression is suppressed by BMP-4, which prevents chondrocyte hypertrophy (Miljkovic et al. 2008). Proliferating chondrocytes that are located close to the perichondrium produce BMP-7 (Medical Advisory Secretariat 2005). MSCs exhibit a reduced capacity for proliferation in the presence of BMP-7, but they produce more cartilage matrix proteins (Danišović et al. 2012). BMP-7 has anti-catabolic activity as well as anabolic activity, which includes suppressing the expression of matrix proteases and cytokines (Brent et al. 2005). In addition to being engaged in promoting joint formation, GDF-5, also identified as BMP-14, is identified during the early condensation stage (Erlacher et al. 1998). It has been demonstrated that GDF-5 promotes chondrocyte maturation and mesenchymal cell survival. Different BMP isoforms have both overlapping and unique

roles, and as a result, their involvement in the levels and phases of cartilage growth varies (Takahara et al. 2004; Tsumaki et al. 1999).

6.4.3 SOX-9 Signaling Pathway

SOX-9 (SRY-Related HMG Box Gene 9) controls chondrocyte differentiation and cartilage synthesis among the various transcription factors responsible for the growth of cartilage. SOX9, which binds to the SOX-9 regulator sites, causes the upregulation of several cartilage-related genes, including aggrecan and the collagen type II, IX, and XI genes (Demoor et al. 2014). During chondrogenesis, SOX-9 is required for MSC condensation. In SOX-9-expressing cells, aggregation occurs, and chondrocyte lineage differentiation begins (Demoor et al. 2014). Depletion of SOX-9 in limb buds causes the mesenchyme condensation to disrupt, preventing the development of cartilage and bone (Akiyama et al. 2002). Therefore, SOX-9 is considered as crucial in mesenchymal condensation-related chondrocyte differentiation. However, its inactivation following condensation phase resulted in serious chondrodysplasia, decreased chondrocyte multiplication, and aberrant joint development (Akiyama et al. 2002). The transcription factors SOX-5 and SOX-6 are also known to direct MSCs toward the chondrogenic lineage. When coupled with SOX-9, they activate type II, IX, and XI collagens along with aggrecan (Smits et al. 2001). By producing scleraxis (SCX), a tendon and ligament transcription factor, chondroprogenitors change their fate away from tendon and ligament lineage in the absence of SOX-5 and SOX-6 (Brent et al. 2005). Mild skeletal abnormalities and chondrodysplasia are caused by mutations in either one or both genes, respectively (Brent et al. 2005). The process of endochondral ossification is another process in which SOX-9 is involved in addition to its function in stimulating chondrogenesis. When SOX-9 is deleted or removed, juvenile chondrocytes develop into hypertrophic cells, whereas excessive upregulation of SOX-9 inhibits the pathways of chondrocyte enlargement in immature chondrocytes (Bi et al. 2001). In addition to being involved in chondrogenesis, SOX-9 is also responsible for the development of the growth plate (Hattori et al. 2010; Zhou et al. 2006; Leung et al. 2011). SOX-9 has been demonstrated to inhibit RUNX-2 activity and to reduce genes produced by hypertrophic chondrocytes, including type X collagen and VEGF-A (Furumatsua and Asahara 2010). It has been demonstrated that the anterior–posterior limb axis patterning molecule sonic hedgehog and the positive promoter of chondrogenesis hypoxia-inducible factor 1 trigger the transcriptional activation of SOX-9 and increase its production (Furumatsua and Asahara 2010). Moreover, the markers TGF- β and BMP-2, together with FGF-1/2 and IGF-1, are also thought to be involved in promoting the expression of SOX-9 (Furumatsua and Asahara 2010). Therefore, in order to govern the growth of cartilage, SOX-9 can connect with the signaling pathways that other molecules have activated or controlled (Fig. 6.4).

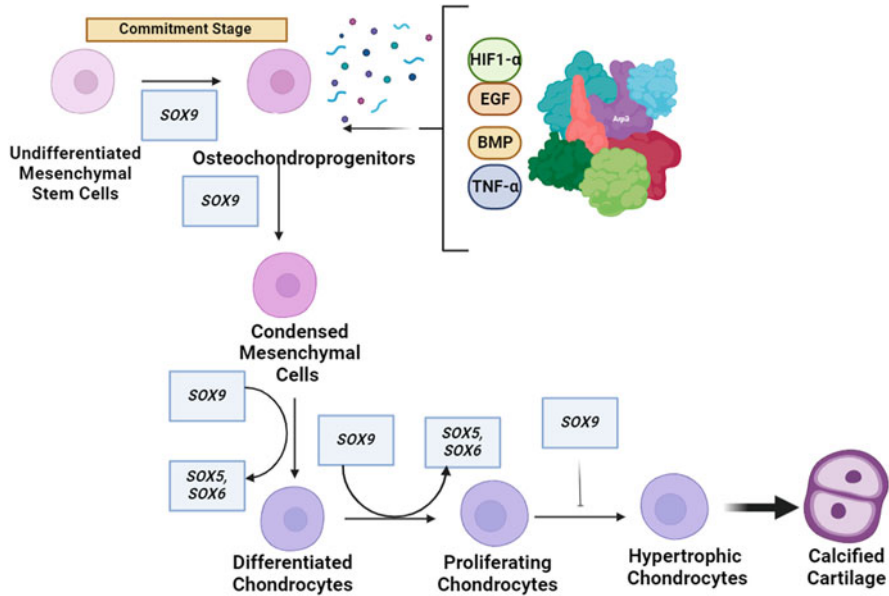


Fig. 6.4 SOX-9 signaling pathway involved in cartilage formation: Mesenchymal cells are committed to osteo-chondroprogenitors by SOX9 in the lateral plate mesoderm. During chondrogenic mesenchymal condensation, following observable chondrocyte maturation, and regular chondrocyte multiplication, SOX9 is also required. SOX5 and SOX6 expression depends on SOX9 and is at least partially responsible for apparent chondrocyte development and proper chondrocyte proliferation. Last but not the least, SOX9 prevents chondrocytes from transforming into hypertrophic tissue as they proliferate

6.4.4 IGF Signaling Pathway

IGF (insulin-like growth factor) performs a variety of roles in the development of cartilage. IGF is essential for the differentiation of mesenchymal cells into chondrocytes as well as for later phases of development, like the production of cartilaginous matrix. Additionally, IGF works with the phosphoinositide 3-kinase (PI3K) pathway, which includes ERK, p38 kinase, and protein kinase C (PKC) signaling, to promote mesenchymal cell chondrogenesis as well as the preservation and survival of differentiated articular chondrocytes (Oh and Chun 2003). Furthermore, it was shown that the association between chondrogenesis and osteogenesis depends heavily on IGF. Particularly, it was discovered that IGF-1 increased the expression of chondrogenic markers such as type II collagen and SOX-9 and induced cell proliferation in MSC pellets (Longobardi et al. 2006). During the chondrogenic growth of MSCs, IGF seems to act individually; however, its actions can be amplified when coupled with TGF- β or BMP-2 (Longobardi et al. 2006). During the initial stages of chondrogenesis, IGF serves as an essential element for chondrocyte proliferation (Demoor et al. 2014). IGF-1 primarily affects SOX-9 and

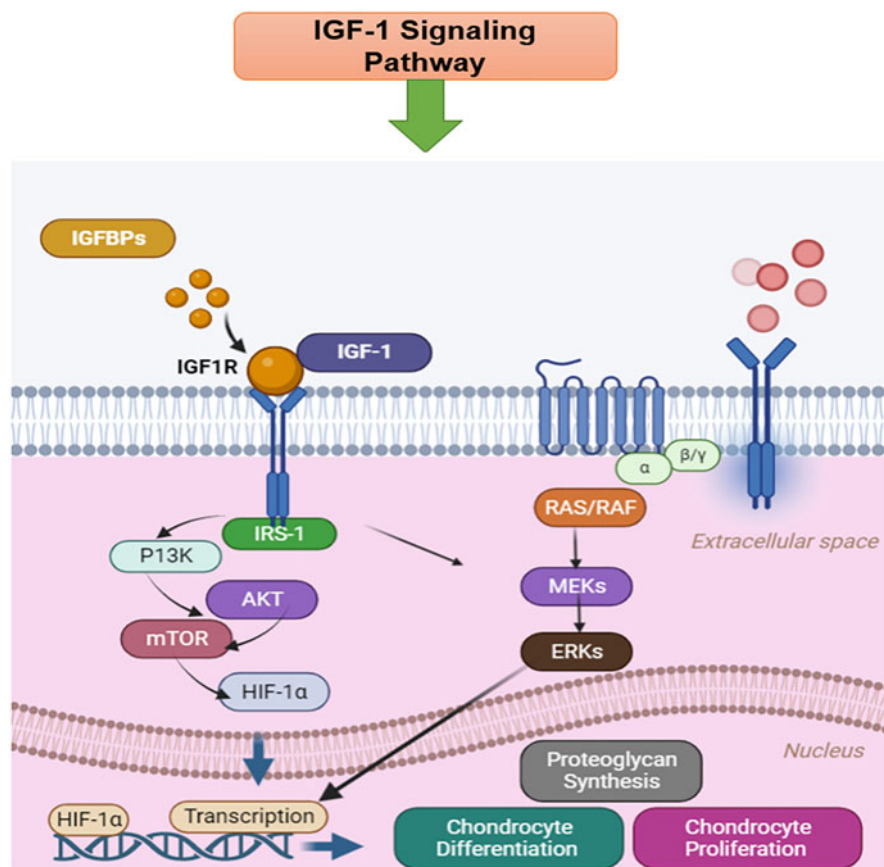


Fig. 6.5 IGF signaling pathway in cartilage development. IGF-1 promotes MSC differentiation into chondrocytes. IGF-1 enhances chondrocyte multiplication by stimulating the PI3K and MEK/ERK networks and enhances glycosaminoglycan production in chondrocytes

specificity protein (Sp1/Sp3) binding to their *cis* elements at the intron-specific enhancer region of type II collagen gene, which necessitates a physical contact with p300 (Karsenty et al. 2009). IGF was found to be essential for the hypertrophic maturation of chondrocytes, which is a process distinct from chondrogenesis (Wang et al. 2011b). The discovery, that hypertrophic chondrocytes could transform into osteoblasts and osteocytes during the emergence of endochondral bone as well as bone regeneration, challenged the conventional perception that chondrocytes and osteoblasts are completely separate lineages descending from a common progenitor (Karsenty et al. 2009; Wang et al. 2011b) (Fig. 6.5). The important significance of IGF as a controller of the mentioned processes is supported by the different cellular gene expression patterns that exist for the IGF pathway through both chondro- and osteogenesis (Wang et al. 1995). In addition, hypertrophic chondrocytes have high levels of IGF receptor mRNA expression (IGF-1R and IGF-2R). Because IGF and

IGF receptors react identically to growth plate chondrocytes, IGF plays a role in the development of adult chondrocytes as well as osteoblasts. IGF is essential for the growth plate's chondrocyte development (Demoor et al. 2014). In an in vitro experiment using chondrocytes from growth plates of embryonic and postnatal mice lacking IGF-1R gene, enhanced PTHrP expression was observed, which results in delayed cell division and accelerated cell death (Wang et al. 2011b). Thus, IGF plays a variety of roles during the early stages of chondrogenesis as well as in hypertrophy and the formation of new connective tissue. When combined with TGF- β 1, IGF-1 increased the overall formation of cartilage compared to IGF-1 alone (Fukumoto et al. 2003). It has been observed that TGF- β acts early to stimulate chondrogenesis, but IGF-1 intensified and maintained proliferation, increasing the overall development of cartilage. Similarly, IGF-1 induces chondrogenic differentiation of MSCs, but more so when combined with TGF- β 1 (Longobardi et al. 2006; Worster et al. 2001). With advancing age and osteoarthritis, the capacity of chondrocytes to respond to IGF-1 declines. Evidence points to decoupling of IGF-1 responsiveness in OA, demonstrating that in OA cartilage organ cultures, IGF-1 may strongly promote proteoglycan production at saturating levels but is unable to influence proteoglycan catabolism (Fortier and Miller 2006; Loeser et al. 2000, 2002; Martin et al. 1997; Doré et al. 1994; Morales 2008). By boosting matrix synthesis, IGF-1 and BMP-7 together have a better ability to promote repair than either growth factor alone (Chubinskaya et al. 2007).

6.4.5 FGF Signaling Pathway

FGFs (fibroblast growth factors) are a group of 22 structurally similar proteins with associated functional and biological characteristics (Kwon et al. 2016). Specific mutations in the genes that encode FGFRs 1, 2, and 3 are etiologically connected to two important categories of skeletal developmental disorders. FGFR activity during the initial stages of chondrogenesis is crucial for limb development (Kwon et al. 2016). The establishment of the apical ectodermal ridge depends on FGFR2b receiving signals from mesenchymal-expressed FGF-10. A form of reciprocal signaling between FGFR1c in the limb mesoderm and FGF-8 in the apical ectodermal ridge is initiated (Ornitz and Marie 2002). FGFR1 is expressed during the mesenchymal condensation stage in the condensing mesenchyme ectoderm, whereas FGFR2 is present at the mesenchymal condensation periphery (Ellsworth et al. 2002). Despite being the most often used growth factor in the development of cartilage tissue, chondrogenesis is still largely unexplained. Since FGF-2 has been demonstrated to inactivate signaling pathways involving IGF-1 and TGF- β , this may be the result of its indirect regulatory activity during chondrogenesis (Ellsworth et al. 2002; Peters et al. 1992). The differentiation and proliferation of chondrocytes trigger initiation of FGFR3 expression during chondrogenesis. Chondrogenesis is also aided by other members of the FGF family (Peters et al. 1992; Deng et al. 1994). In mouse primary costal chondrocytes, it has been demonstrated that FGF-1, FGF-2, and FGF-7 increase SOX-9 expression through the MAP kinase/ERK1/2 cascade

(Murakami et al. 2000). In addition to FGF-1, FGF-2, and FGF-3, other FGF family members, including FGF-9, FGF-10, and FGF-18, are expressed in later phases of chondrogenesis (Peters et al. 1992). The key regulatory influence of FGF-8 in heterotopic ossification, a kind of endochondral ossification, validated central function of FGF-8 in chondrogenesis (Valta et al. 2006). Tyrosine kinase receptors of the FGF family mediate their actions, and their activation triggers a mitogenic response in multiple cell types, including chondrocytes (Raucci et al. 2004). FGFs and FGFRs both contribute a significant impact in chondrocyte hypertrophy; in particular, FGFR1 is expressed in both pre- and post-hypertrophic chondrocytes and promotes the survival and maintenance of hypertrophic chondrocytes (Luyten et al. 1988). Additionally, hypertrophic chondrocytes express FGFR3 (Davidson et al. 2005). The expression of FGFR1, which was first identified in chondrocytes generated from condensation of mesenchymal stem cells, is largely observed in chondrocytes in the peripheral mineralized region and in the adjacent osteoblasts, suggesting a potential role in osteoblastic growth and hypertrophy (Delezoide et al. 1998). However, FGFR3 is mostly expressed in the dividing chondrocytes, suggesting its regulatory function in chondrocyte development (Delezoide et al. 1998). Through FGFR3 signaling, FGF-18 has been found to regulate primary stage chondrocyte multiplication and differentiation (Liu et al. 2007). Surprisingly, FGF-9 may also contribute to chondrocyte growth and hypertrophy. Only FGF-9 and FGF-18 have been determined to be active growth factor in the chondrocyte hypertrophy phase, despite the fact that several FGF ligands participate in all processes of cartilage tissue formation. It has been demonstrated that during chondrogenesis, various subtypes of FGF growth factors involved in signaling pathways. Signaling interaction between members of FGF, TGF- β , and Wnt protein families regulates various stages of differentiation during chondrogenesis (Pogue and Lyons 2006; Cleary et al. 2015). The SOX-9-dependent chondrocyte-specific enhancer regions in the type II collagen gene are activated by FGFs, increasing SOX-9 expression levels (Murakami et al. 2000). It is obvious that several signaling pathways work well together to induce chondrogenesis (Fig. 6.6).

6.4.6 Wnt/ β -Catenin Signaling Pathway

The growth of bone and cartilage is regulated by a broad family of 19 secreted glycoproteins known as Wnts (Wingless-type) (Staines et al. 2012; Ma et al. 2013; Sharma et al. 2013; Sassi et al. 2014; Bhanot et al. 1996). Wnt signaling also contributes to the communication between subchondral bone and cartilage. The amount of β -catenin expressed by chondrocytes in the lower hypertrophic zone influences the rate of trabecular bone production both during late embryonic development and after birth. Wnts have long been acknowledged as important controllers of joint and bone formation and homeostasis (Chun et al. 2008; Nalesso et al. 2011; Golovchenko et al. 2013; Dell'Accio et al. 2006). The generation of RANKL in hypertrophic chondrocytes must be regulated in order to regulate osteoclastogenesis in subchondral growth plates (Nusse 2005). It is plausible to suppose that if OA

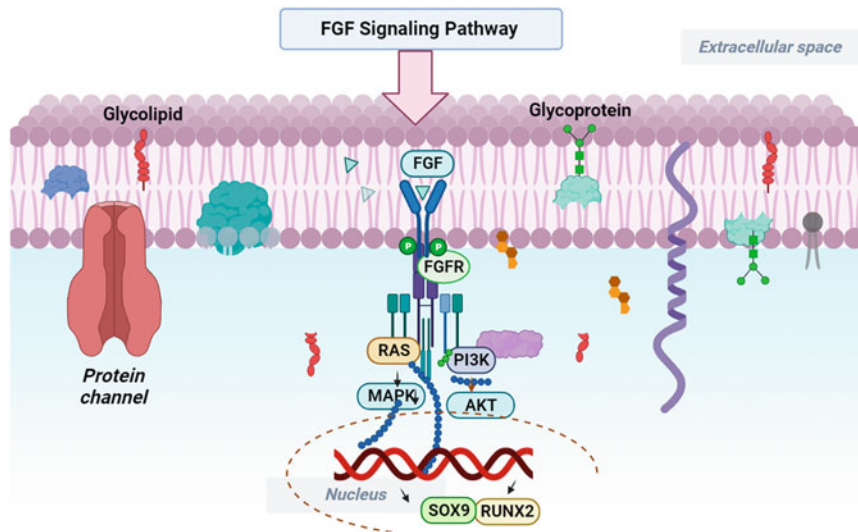


Fig. 6.6 FGF signaling cascade in the development of cartilage. FGF and FGFR work together to initiate the downregulation of the cascade (RAS-MAPK and PI3K-AKT), which regulates the transcription of SOX9 and RUNX2. In the maturation of cartilage, FGF can inhibit BMP/Smad signaling via MAPK signaling

worsens, the observed modified activity of Wnt signaling in chondrocytes may have an impact on the osteoclastic activity in subchondral bone growth plates, leading to the development of sclerosis or osteophytes at the borders of joints (Chun et al. 2008). As in the case of OA, the altered Wnt signaling pathway in chondrocytes seems to control significant regulator variables for remodeling of subchondral bone. In a similar manner, aberrant Wnt signaling in subchondral bone may modify chondrogenic factors that are essential for preserving cartilage homeostasis (Nalesso et al. 2011). In context of this research, scientists are now looking into possible links between Wnt changes and OA. In both human OA cartilage and damaged cartilage, elevated canonical Wnt pathway activation has been observed (Dell'Accio et al. 2006, 2008; Eltawil et al. 2009). Additional research on animal models reports a connection between activation of the β -catenin signaling pathway and an OA-like phenotype (Lodewyckx et al. 2012) (Fig. 6.7).

6.4.7 Growth Differentiation Factor 5 (GDF5) Signaling Pathway

GDF5, a constituent of the TGF- β group of protein, was initially identified as the marker for brachypodism in rodents with aberrant skeletal architecture, particularly in proximal joints (Storm et al. 1994). Brachydactyly and chondrodysplasia are skeletal deformities caused by mutations of the human GDF5 gene. Brachydactyly is also a result of mutations of the gene that codes for the GDF5 receptor, BMPR1B

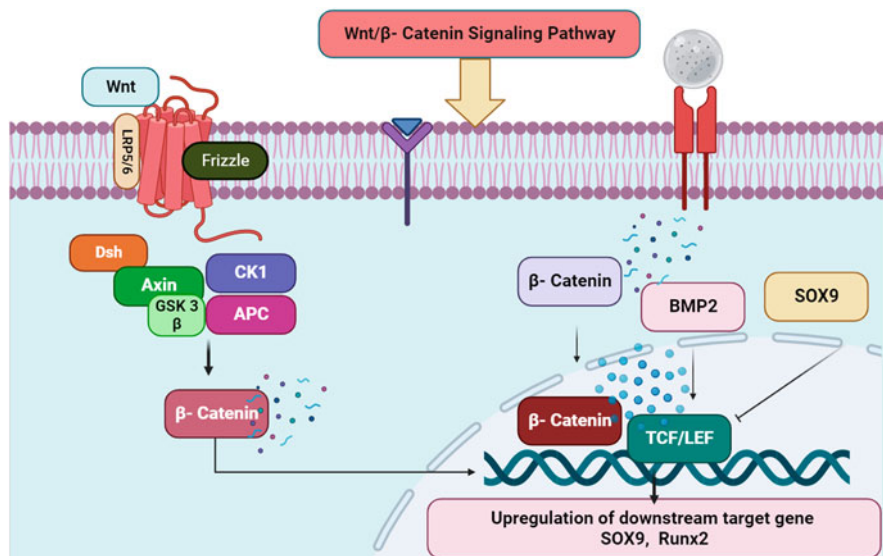


Fig. 6.7 The Wnt/β-catenin signaling pathway in cartilage development. Wnt ligands interact with Frizzled and LRP5/6 to recruit Dsh once Wnt signaling is functional, which enhances the concentration of cytoplasmic β-catenin. The activation of the target genes that is Runx2 and Sox-9 is therefore controlled by β-catenin entry into the nucleus and binding to TCF/LEF

(Yi et al. 2000; Baur et al. 2000). According to these findings, GDF5 is crucial for the formation of healthy cartilage structure. Gdf5 transcripts are initially detected sporadically in the proximity of the pre-cartilage region. Later, it is stronger and restricted to the interzone (Francis-West et al. 1999; Storm and Kingsley 1999; Hartmann and Tabin 2001). The fusion of joints occurs in brachypoditic mice because GDF5 is highly expressed throughout the cartilage anlagen and outside of the interzone (Storm and Kingsley 1999). According to these facts, GDF5 plays a role in developing and maintaining the interzone. Some proximal joints, such as the elbow and knee joints, are not fused in brachypodism mice, despite the fact that GDF5 is found in the majority of synovial joints of the limbs (Harada et al. 2007). GDF6, a different member of the GDF family that is primarily expressed in proximal joints, likely compensates for this consequence (Wolfman et al. 1997). In fact, significant joint deformity caused by twin mutations in GDF5 and GDF6 is not seen in either mutant (Storm and Kingsley 1996; Settle et al. 2003).

According to transcriptional expression of GDF-5, prechondrogenic condensation is essential for chondrogenesis (Buxton et al. 2001). Researchers have found evidence in living organisms that genetic changes in GDF5 may play a role in autosomal recessive disorders like Grebe-type and Hunter–Thompson chondrodysplasia in humans and brachypodism (bp) in mice. The skeleton is thinned in these diseases, and the growth and development of the cartilage is aberrant (Storm and Kingsley 1996; Thomas et al. 1996, 1997). For a deeper comprehension of how

GDF5 controls skeletogenesis, numerous *in vivo* molecular function studies have been conducted. According to the research, adequate production of GDF5 in growing chick limbs and embryos increased the size of the skeletal muscle at the condensation step or afterward, when the skeletal components have been produced, accordingly (Buxton et al. 2001). GDF5 overexpression enables chondrogenesis, which enhances mesenchymal cells' ability to adhere to one another and the development of chondrocyte (Francis-West et al. 1999). The presence of GDF5 during mesenchymal condensation promoted N-cadherin activity and further enhanced cell-cell adhesion, hence increasing condensation (Coleman and Tuan 2003). MSCs are frequently studied in the context of *in vitro* chondrogenic differentiation employing a pellet culture technique in which MSCs are concentrated to resemble mesenchymal condensation (Francis-West et al. 1999). The potential of bone marrow-originated mesenchymal cells to distinguish into chondrocytes structures in chondrogenic MSC pellets can be markedly improved with the addition of recombinant human GDF5 protein (rhGDF5), as demonstrated by the production of collagen type II and sulfated glycosaminoglycan (GAG) into the extracellular matrix (ECM). These cultures' maturation and hypertrophy were also facilitated by GDF5 (Yoo et al. 1998; Coleman et al. 2013). In three-dimensional aggregate culture, GDF5, TGF-1, and BMP-2 significantly upregulate the chondrocyte genes in human MSCs. It is interesting to note that after 4 weeks of growth, this combination leads to the highest overexpression of chondrocyte genes, namely SOX-9, COL2A1, and ACAN, as well as the production of cartilage-specific matrix. Strong cartilage rich in GAGs and collagen type II was generated as a result (Murphy et al. 2015).

Recombinant GDF5 protein can also enhance chondrogenic development in stem cells, including canine, chick, and rabbit adipose-derived stromal cells, canine MSCs, and fetal human MSCs (Han et al. 2016). Additionally, chondrogenesis in adipose stem cells is impacted by GDF5 overexpression caused by an adenovirus (Feng et al. 2008). The TGF- β signaling protein Smad 1/5/8, which is phosphorylated, has a significant impact on the last step of chondrocyte differentiation (Miyazono et al. 2010). The GDF5 activity improved both the chondrogenic differentiation of these cells and their hypertrophic differentiation, which led to Smad 1/5/8 phosphorylation in chondrogenic MSC pellets (Coleman et al. 2013). p38 mitogen-activated protein kinase (MAPK) and transcriptional regulator Trps1 are also engaged in GDF5-involved chondrocyte differentiation. Growth/differentiation factor 5 has a functional involvement in the limb mesenchymal cells' chondrogenesis (Coleman and Tuan 2003; Nakamura et al. 1999). In the chondrogenic cell line ATDC5, GDF5 increased phosphorylation of p38 and nuclear translocation of Trps1, as well as further elevated COL2A1 gene expression. The p38 signaling inhibitor SB203580 prevented these effects. Additionally, ATDC5 cells that overexpressed Trps1 were more likely to develop into chondrocytes (Nakamura et al. 1999; Itoh et al. 2008). Trps1 and p38 work as descending regulators of GDF5 signal transduction pathway to aid in the growth of chondrocytes. GDF-5 is essential for chondrogenesis, which controls the growth of bone and cartilage (Itoh et al. 2008) (Fig. 6.8).

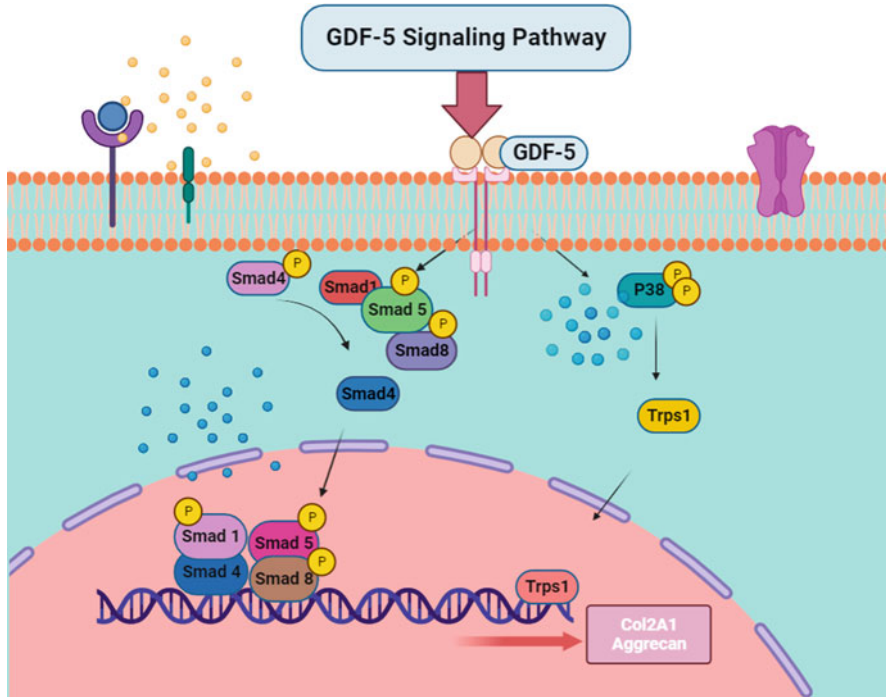


Fig. 6.8 The GDF-5 signaling pathway in cartilage development. Smad4 participates in the formation of a complex with Smad1 when GDF5 activates Smad1/5/8 by binding to its receptors. The complex enters the nucleus, where it regulates the expression of its target genes, including COL2A1 and ACAN. In addition, GDF5 has the capacity to phosphorylate p38, which promotes Trps1 nuclear translocation and increases the expression of the COL2A1 gene

6.4.8 Hedgehog Signaling Pathway

Hedgehog signaling pathway is spatially and temporally controlled during the formation of endochondral cartilage and bone, controlling how growth plate chondrocytes differentiate. In the growth plate, Indian hedgehog (IHH) is the primary hedgehog ligand, expressed at high expression in pre-hypertrophic chondrocytes (Vortkamp et al. 1996). IHH causes ossification of the perichondrium around the chondrocytes and prevents chondrocyte development to a hypertrophic state. IHH is exclusively necessary for the development of endochondral bone (Karp et al. 2000). Additionally, IHH can promote chondrocyte maturation into a hypertrophic state by enhancing the production of PTHLH, which transcribes a parathyroid hormone-related protein (Lanske et al. 1996). Growth plate chondrocytes cannot differentiate into hypertrophic cells because PTHrP expression keeps them in a proliferative state (Minina et al. 2001). A PTHrP concentration gradient likely plays an important role in how growth plate chondrocytes escape this proliferative

state, but the exact mechanism is yet unclear (Minina et al. 2002). The negative feedback loop formed by IHH and PTHrP regulates and synchronizes the rate of chondrocyte differentiation in the growth plate (Vortkamp et al. 1996). Early development is characterized by the production of PTHrP by periarticular cells, which appear to vary from chondrocytes at later embryonic stages, while in the postembryonic growth plate, proliferating chondrocytes appear to be a source of PTHrP. BMPs, which also play a role in the beginning of chondrocyte development, are implicated in the regulation of PTHrP expression by IHH (Lanske et al. 1996; Minina et al. 2001). In mice lacking IHH, chondrocyte growth is reduced, the hypertrophic region in the development stage is increased, and endochondral ossification of the appendicular skeleton is not observed (Lanske et al. 1996; Minina et al. 2001). A similar extended hypertrophic zone and decreased bone growth are observed in mice embryos lacking GLI2, where there is a downregulation in *Ptch1* and *Pthlh* expression levels, associated with a reduction in hedgehog signaling (Miao et al. 2004; Mau et al. 2007).

IHH-mediated signaling in growth plate chondrocytes is thought to be negatively regulated by GLI3, as evidenced by the fact that the genomic elimination of GLI3 reverses the pattern caused by the loss of IHH (Mau et al. 2007; Koziel et al. 2005). In addition, PTHrP also controls the development of growth plate chondrocytes in a hedgehog ligand-independent and protein kinase A-dependent manner, partially via the activity of GLI3 (Mau et al. 2007; Epstein et al. 1996; Riobó et al. 2006). The hedgehog ligand repressor function of GLI proteins, however, did not participate in chondrocyte or osteoblast distinctions, as demonstrated by a comprehensive phenotypic examination of complex mutant mouse strain. According to this observation, the management of GLI protein transcriptional regulation is crucial for endochondral ossification (Holtz et al. 2013) (Fig. 6.9).

6.5 Late-Stage Signaling Pathways Involved in Cartilage Development

6.5.1 *Prg4* Signaling Pathway

The synovial fluid, which is formed by articular chondrocytes and synoviocytes, contains one of the main components, lubricin, which is transcribed by *Prg4* (Proteoglycan 4) gene (Coles et al. 2010; Schumacher et al. 1994). Joint lubricity is caused by the protein lubricin, whose expression is diminished in osteoarthritis. Osteoarthritis may improve from exogenous lubricin injections (Hughes et al. 2005; Flannery et al. 2009; Bao et al. 2011). At the beginning of joint cavitation, *Prg4* expression is observed (Bhanot et al. 1996). Even after cartilage development and maturation, lubricin is still primarily produced in the synovium and articular cartilage surface cells (Yamagata et al. 1989). It has been discovered that tensile load, PTHrP, and TGF- β are all upstream regulators of *Prg4* (Matsuzaki et al. 2018; Hill et al. 2015). When the canonical Wnt signaling is temporarily activated, it increases SFZ cell growth and *Prg4* expression, and when it is deleted, SFZ formation is

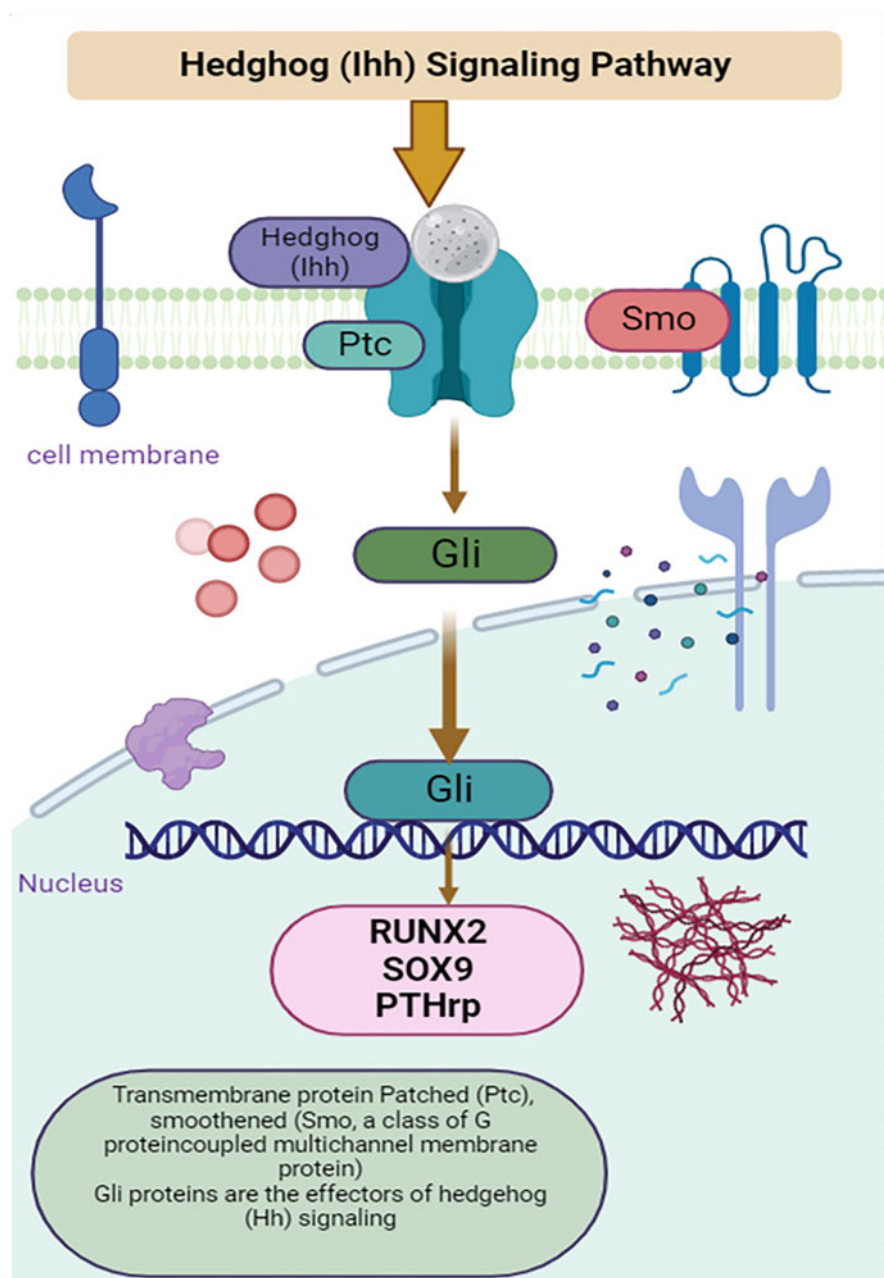


Fig. 6.9 The Ihh signaling pathway in cartilage development. The generation of the Ihh signal allows its amino-terminal portion to interact with the Patched (Ptc), which prevents it from being suppressed. As a result, Smo is triggered. The response was then sent to downstream target genes, activating the GLI family of genes. Finally, GLI enters the nucleus to control the transcription of the target genes (Sox-9, Runx2, PTHrp)

hindered along with Prg4 downregulation (Yasuhara et al. 2011). Acellular layer deposition with aging induces abnormalities at the surface of the articular cartilage in mice with Prg4 deletion, which does not affect skeletal development in the neonatal period (Coles et al. 2010; Hill et al. 2015). Despite Prg4 is present at the joint cavitation, it presumably has a smaller impact on joint formation and aids in articular cartilage maintenance.

6.5.2 Notch Signaling Pathway

An additional evolutionary conserved mechanism, Notch signaling, is implicated in cell fate determination, differentiation, proliferation, and death (Baldrige et al. 2010). When the Notch receptor binds to the ligands Delta or Jagged, which are type 1 transmembrane proteins anchored on neighboring cells, it results in the activation of the Notch receptor (Engin and Lee 2010). Following disintegration, the Notch receptor produces a component known as notch extracellular truncation (NEXT). In order to produce the notch intracellular domain, this fragment is further cleaved by γ -secretase proteinase (NICD). RBPJ and the transcriptional coactivator Mastermind-like 1 (MAML1) are both involved in NICD translocation to the cell nucleus for interaction. Basic helix loop-helix (bHLH) transcription factors of the HES and HEY families are expressed more readily by the ternary complex, which then causes their target genes to undergo transcriptional repression (Samsa et al. 2017).

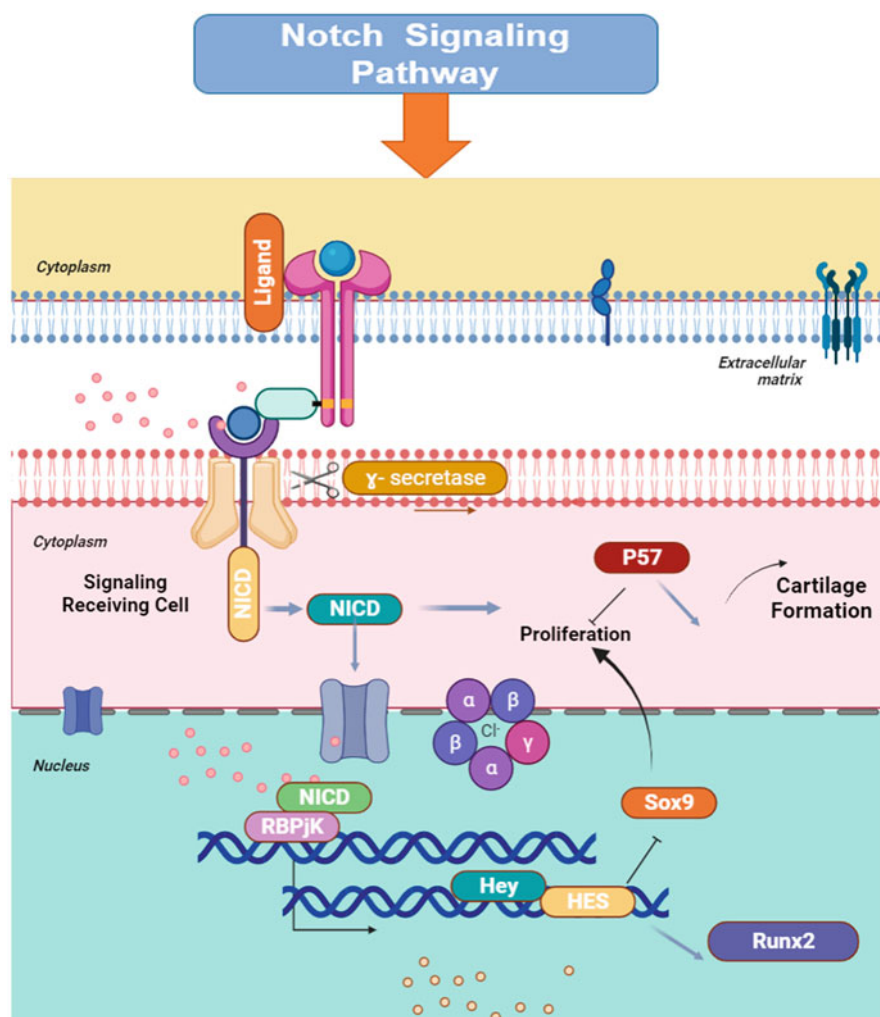
The development of cartilage and bones is significantly influenced by Notch signaling. Numerous abnormalities in patterning, axial skeleton development, and skeletal disorders are linked to mouse genetic deletions and human genetic mutations in Notch signaling (Samsa et al. 2017; Chen et al. 2014). Through the control of Sox-9 expression, Notch signaling is crucial for chondrocyte hypertrophy (Engin and Lee 2010; Kohn et al. 2015). Notch functions upstream of Sox-9 and Runx2 in the maintenance of osteochondro-progenitor cell proliferation and lineage specification, according to mouse models with loss and gain of Notch signaling function (Kohn et al. 2015). Furthermore, the deletion of Notch activity in these cells increased the extent of the hypertrophic region; meanwhile, the overexpression of NICD exclusively in chondrocytes (Col2a1Cre and Col2a1CreER) led to skeletal deformities with reduced multiplication and delayed chondrocyte hypertrophy (Mead and Yutzey 2009; Chen et al. 2013). By inhibiting the expression of Sox-9, Notch indirectly controls chondrocyte development in the skeletal system (Chen et al. 2013).

An RBPJ k-dependent pathway that has been connected to the downregulation of chondrocyte development, survivability, and columnar organization as well as decreased response to Ihh signaling is responsible for facilitating the development of chondrocytes (Rutkowski et al. 2016). Similar to this, the role of Notch signaling in growth plates is much more complex since it partially regulates chondrocyte hypertrophy without the help of Sox-9 (Kohn et al. 2015). Additional evidence that deletion of Notch effectors in osteochondral progenitors speeds up chondrocyte

development comes from *Prx1*-Cre loss of *Hes1* and *Hes5*, which are the main substrates of RBPj-dependent Notch signaling (Rutkowski et al. 2016). *Hes5* individually regulates the expression of *SOX9*, even though *Hes1* and *Hes5* collaborate to reduce chondrogenesis and favor hypertrophy. Likewise, the upregulation of *Hes1* in osteochondral progenitor cells delays and inhibits chondrocyte enlargement and multiplication. In contrast, the *HEY* family of transcription factors has no effect on either chondrogenesis or matrix degradation, and *Hes1* and *Hes5* are not required for endochondral ossification in chondrocytes (Karlsson et al. 2010). This emphasizes how Notch signaling contributes to growth plate homeostasis at particular stages. Notch may prevent limb bud mesenchyme from differentiating into cartilage by increasing the expression of *Twist1*, which is a gene encoding basic helix loop-helix (bHLH) transcription factor important for skeletal development (Tian et al. 2015). These studies suggest that regulation of the transcription of major cell fate controllers like *SOX9* and *Twist1* is one of the key roles of Notch signaling in chondrogenesis and growth plate development. Understanding the downstream Notch targets also provides insight into the normal and abnormal functioning of Notch in skeletal diseases (Fig. 6.10).

6.6 Conclusion

Growth plate production, a specialized and dynamic cartilage tissue, is necessary for the development of the vertebrate skeleton as well as for growth and endochondral new bone formation. Key elements of growth plate development are closely related to major signaling pathways that are triggered by classical morphogens as well as by additional systemic and tissue-specific stimuli. In this chapter, we discussed key elements and the signaling cascades involved in cartilage growth. Over many years, a lot of research has been focused on the molecular processes that underlie the development of cartilage and endochondral ossification. Growth plate development is a dynamic, organized, and carefully regulated process. Numerous signaling pathways collaborate to play crucial roles in the process. In recent years, research is also focused on the pathophysiology of osteoarthritis and the homeostasis of articular cartilage. In addition, this chapter covers the chemicals, signaling pathways, and cells that control early and late stages of articular cartilage development and maturation. Additionally, articular cartilage formation in humans may differ significantly from that in mice, where it is both thinner and multilayered. As a result of these crucial roles, devastating skeletal diseases result when these signaling mechanisms are disrupted by mutations or environmental causes. Here, we examined these mechanisms together with the current findings of how chondrocyte differentiation affects growth plate development and activity.



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Role and Application of Biomolecules for Regeneration of Cartilage Tissue

7

Ryo Nakamichi, Yuta Fujii, and Hiroshi Asahara

Abstract

To date, many studies have been conducted on the developmental process and homeostatic mechanism of articular cartilage. The major goal of these studies is to apply these findings to develop therapy that activate the intrinsic cartilage-forming or tissue-maintaining abilities of cells. In this chapter, we review the detailed molecular mechanism in the following subsections: 1.1. articular cartilage component, 1.2. signaling pathway, 1.3. transcription factors, 2.1. RNA-binding proteins, 2.2. microRNA, 2.3. circular RNA, 2.4. ubiquitination, and 2.5. mitochondrial mechanisms. We hope that understanding current knowledge and missing points will help us to realize ideal cartilage tissue regeneration therapy.

Keywords

Articular cartilage regeneration · Articular cartilage development · Biomolecules

Ryo Nakamichi and Yuta Fujii contributed equally with all other contributors.

R. Nakamichi

Department of Orthopaedic Surgery, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan

Department of Molecular Medicine, Scripps Research, La Jolla, CA, USA

Department of Systems Biomedicine, Tokyo Medical and Dental University, Tokyo, Japan

Y. Fujii · H. Asahara (✉)

Department of Molecular Medicine, Scripps Research, La Jolla, CA, USA

Department of Systems Biomedicine, Tokyo Medical and Dental University, Tokyo, Japan

e-mail: asahara@scripps.edu

7.1 Introduction

Osteoarthritis (OA) causes motor dysfunction. Its progression is characterized by the loss of joint function and progressive destruction of the articular cartilage (AC). The homeostasis of AC is maintained by the ability of chondrocytes to produce the extracellular matrix (ECM), type II collagen, proteoglycans, and other components. However, once damaged, normal cartilage function is difficult to recover due to its low self-regeneration potential. To overcome this problem, studies have increasingly focused on the developmental and homeostatic mechanisms of AC. The major goal of this research was to understand these mechanisms and facilitate improvements in OA therapies.

In the early stages of AC development, a skeletogenic process known as endochondral ossification occurs. This process begins with the condensation of mesenchymal cells, following which the chondrocyte primordium grows with proliferation and differentiation, resulting in a shape similar to that of the future bone. In the center, chondrocytes differentiate into hypertrophic chondrocytes, which is followed by apoptosis, vascular invasion, and ossification by osteoblasts. This sequence of events extends longitudinally to the diaphysis, which is referred to as the primary ossification center. Subsequently, another ossification site, the secondary ossification center, forms at the epiphysis. Part of the cartilage between these two ossification centers remains as a growth plate during growth, and the area between the joint cavity and secondary ossification center becomes AC (Long and Ornitz 2013; Nakamichi et al. 2020). After cartilage formation is complete, AC homeostasis is maintained by various mechanisms.

In this chapter, we review the detailed molecular mechanism in the following subsections: 1.1. AC component, 1.2. signaling pathway, 1.3. transcription factors, 2.1. RNA-binding proteins, 2.2. microRNA, 2.3. circular RNA, 2.4. ubiquitination, and 2.5. mitochondrial mechanisms. We hope that this chapter will promote the development of new approaches for cartilage regeneration.

7.2 Biomolecules Affecting Cartilage Development and Maturation

Cartilage development begins with the formation of an interzone (IZ), a region of cohesive precartilaginous tissue, shortly before the appearance of the cartilage primordium (Mitrovic 1977; Ito and Kida 2000). The IZ appears within the precartilaginous tissue, and the cartilage primordium divides around it (Ito and Kida 2000). The IZ is necessary for skeletal element segmentation and articular formation; these roles were verified by the formation of long bones without joints upon removal of the IZ from a chick embryo (Holder 1977). The IZ is composed of three layers, namely two dense outer layers of round cells and an inner layer of flat cells. The outer layer contributes to the bony ends of long bones, whereas the inner layer forms the articular surface (Ito and Kida 2000). This morphology suggests that IZ cells are a population of crucial precursor cells necessary for the formation of

mature articular tissue. The formation of the synovial joints precedes that of the joint cavity. In mice, neoarticular joints in the limbs are observed around E12.5–E15.5, whereas AC is identified after birth (Lefebvre and Bhattaram 2010). Growth differentiation factor 5 (GDF5) (Storm and Kingsley 1996, 1999; Rountree et al. 2004; Koyama et al. 2007, 2008; Roelofs et al. 2017; Shwartz et al. 2016; Merino et al. 1999; Francis-West et al. 1999; Tsumaki et al. 2002; Harada et al. 2007; Settle et al. 2003; Rhee et al. 2005; Hartmann and Tabin 2001; Lee and Behringer 2007; Später et al. 2006a, b; Bian et al. 2020), doublecortin (DCX) (Corbo et al. 2002; Zhang et al. 2007a, 2011a; Decker et al. 2014, 2017; Yu et al. 2019; Pitsillides and Beier 2011; Yamagami et al. 2009; Wen et al. 2016; Craft et al. 2013, 2015; Lan et al. 2022), leucine-rich orphan G-protein-coupled receptor (LGR5) (Leung et al. 2018; Feng et al. 2019), Indian hedgehog (IHH) (Storm and Kingsley 1999; Koyama et al. 2007; Später et al. 2006a; Minina et al. 2001; Mak et al. 2006, 2008; Wu et al. 2016; St-Jacques et al. 1999; Baur et al. 2000; Niedermaier et al. 2005; Koziel et al. 2005; Huang et al. 2016; Rockel et al. 2016), and bone morphologic protein (BMP) (Koyama et al. 2008; Merino et al. 1999; Mak et al. 2006, 2008; Ray et al. 2015; Singh et al. 2018a, b; Seemann et al. 2005; Winslow and Burke 2010; Brunet et al. 1998; Wijgerde et al. 2005; Seo and Serra 2007; Garrison et al. 2017; Gelse et al. 2012; Chang et al. 2019a; Gamer et al. 2018) are well known as important factors influencing the early stage of cartilage development. The functions of these factors are summarized in Table 7.1. However, expression of *Gdf5*, *Wnt9a*, *Ihh*, *Bmp*, and *Noggin* disappears during AC maturation (Koyama et al. 2008; Dy et al. 2010; Narendra et al. 2016), and structural proteins, such as lubricin, tenascin-C, CD44, and type II collagen, are expressed (Koyama et al. 2008; Narendra et al. 2016). In addition, TGF- β s, FGF18, and PTHrP are persistently expressed from mesenchymal cells to mature articular chondrocytes and may play a broad role in AC formation. These proteins are considered potential therapeutic targets for OA (Oo et al. 2018). Most ACs are derived from a distinct lineage than that of growth plate cartilage. Postnatal thickening of the AC is primarily characterized by an increase in cell volume rather than cell proliferation or cell death (Decker et al. 2017). The detailed developmental mechanism of AC, such as whether it is derived from the mesenchyme or chondrocyte primordium, has not been fully elucidated. The lack of glycosaminoglycans (GAGs) in the periarticular cartilage of juvenile mice suggests that the AC may be formed by articular chondrocytes (Chijimatsu and Saito 2019). Recent studies suggest that articular components are not solely composed of GDF5-positive cells that form the early IZ but also composed of various cell influxes and outflows during development (Shwartz et al. 2016; Chijimatsu and Saito 2019). *Prg4*-positive cells in the SFZ are precursors of AC (Kozhemyakina et al. 2015), and labeling *Prg4*-positive cells with E17.5 revealed that a lineage of these cells constitutes the entire layer of AC in adults (Kozhemyakina et al. 2015). In this section, we review the cartilage components (Table 7.2), signaling pathways (Table 7.3), and transcription factors (Table 7.4) associated with AC formation.

Table 7.1 Summary of factors influencing the early stage of cartilage development

	Expression lesion	Expression period	Function	References
GDF5	Interzone	Knee, elbow: E11.5–15.5, phalangeal joint: E11.5–neonatal	Promotes cartilage growth	Storm and Kingsley (1996, 1999), Rountree et al. (2004), Koyama et al. (2007, 2008), Roelofs et al. (2017), Shwartz et al. (2016), Merino et al. (1999), Francis-West et al. (1999), Tsumaki et al. (2002), Harada et al. (2007), Settle et al. (2003), Rhee et al. (2005), Hartmann and Tabin (2001), Lee and Behringer (2007), Später et al. (2006a, b), Bian et al. (2020)
Dcx	Mesenchymal precursor	E9.5–12.5	Defines the chondrocyte differentiation potential of GDF5-positive cells	Corbo et al. (2002), Zhang et al. (2007a, 2011a), Decker et al. (2014, 2017), Yu et al. (2019), Pitsillides and Beier (2011), Yamagami et al. (2009), Wen et al. (2016), Craft et al. (2013, 2015), Lan et al. (2022)
Lgr5	Interzone	E13.5–18.5	Chondrocyte progenitor cells that contribute to the formation of joint surfaces	Leung et al. (2018), Feng et al. (2019)

7.2.1 Cartilage Components

7.2.1.1 Type II Collagen (COL2A1)

COL2A1 is robustly expressed in chondrocytes during early embryonic limb development (Cheah et al. 1985). In *Col2-cre; R26R* mice, *Col2*-positive chondrogenic cells lose *Col2a1* expression and begin to express *Gdf5* upon the appearance of the IZ cells (Hyde et al. 2008). *Gdf5*-positive cells influx into the IZ, re-express *Col2a1*, and contribute to the formation of various articular elements, including the AC (Shwartz et al. 2016). In a recent study using *Col2a1-cre; Rosa-td Tomato* mice, almost all chondrocytes of the epiphysis were reported to be positive at postnatal day 4, but the fluorescence intensity was not uniform and was higher in cells present in the superficial layers of the epiphysis. These cells were dense, relatively small, and did not express matrilin-1, which is normally expressed in the epiphyseal cartilage (Kavanagh and Ashhurst 1999). Therefore, these cells may be periarticular cells that

Table 7.2 Summary of components in articular cartilage

Name	Expression	Function	References
Type 2 collagen	Early embryonic limb, articular chondrocyte	Major extracellular matrix (ECM) proteins of growth plates and articular cartilage	Shwartz et al. (2016), Cheah et al. (1985), Hyde et al. (2008), Kavanagh and Ashhurst (1999), Tong et al. (2019)
Aggrecan	Articular chondrocyte	Major extracellular matrix (ECM) proteins of growth plates and articular cartilage	Chambers et al. (2002), Asperberg (2012), Cortes et al. (2009), Mouw et al. (2014), Bishop et al. (2007), Knudson et al. (2000), Miura et al. (1999), Gleghorn et al. (2005), Tompson et al. (2009), Nilsson et al. (2014), Gibson and Briggs (2016), Watanabe et al. (1994), Henry et al. (2009), Rashid et al. (2017)
Lubricin (PRG4)	Articular cartilage surface and synovium	Lubrication reduces joint friction	Koyama et al. (2008), Rhee et al. (2005), Kozhemyakina et al. (2015), Jay et al. (2001), Marcelino et al. (1999), Bahabri et al. (1998), Niikura and Reddi (2007), Ogawa et al. (2014), Matsuzaki et al. (2018), Coles et al. (2010), Hill et al. (2015), Li et al. (2013, 2017), Miyatake et al. (2016), Chavez et al. (2017, 2019), Zhang et al. (2007b, 2011b, 2021a), Jia et al. (2016), Qin and Beier (2019), Wei et al. (2021), Shepard et al. (2013), Pest et al. (2014), Bellini et al. (2020), Akasaki et al. (2014), Eelen et al. (2016), Duffy et al. (2020), Wang et al. (2009, 2020a), Greenblatt et al. (2013), Bedford and Clarke (2009), Tee et al. (2010), Norrie et al. (2016), Ramachandran et al. (2019)
Tenascin-C	Interzone and articular cartilage until maturation of chondrocytes	Differentiation and maturation of chondrocytes during the process of chondrocyte maturation in the embryo and after birth	Jia et al. (2016), Qin and Beier (2019), Hasegawa et al. (2018, 2020), Midwood et al. (2016), Pacifici et al. (1993), Pacifici

(continued)

Table 7.2 (continued)

Name	Expression	Function	References
			(1995), Edwall-Arvidsson and Wroblewski (1996), Pfander et al. (2004), Järvinen et al. (1999), Flück et al. (2008), Namba et al. (1998), Brommer et al. (2005), Gruber et al. (2020)
CD44	Articular surface	Involved in chondrocyte maturation through interaction with Hyaluronic Acid	Bian et al. (2020), Pitsillides et al. (1995), Dowthwaite et al. (1998), Matsumoto et al. (2009), Peterson et al. (2004), Pazin et al. (2014)

contribute to AC development (Tong et al. 2019) and the formation of secondary ossification centers. At P18, osteoblasts and osteocytes in the trabecular bone were derived from these cells with high fluorescence intensity. In addition, pericytes and adipocytes, which could be considered mesenchymal progenitor cells or mesenchymal stem cells, were also present (Tong et al. 2019). Furthermore, when these cells were compared to cells with low internal fluorescence intensity in vitro, the expression of chondrocyte markers, including *Sox9*, *Col2a1*, and *Col10a1*, was low in these cells, and most of these cells were undifferentiated with high proliferative potential (Tong et al. 2019).

7.2.1.2 Aggrecan (ACAN)

ACAN is the major ECM protein found in growth plates and AC. GAG chains, consisting primarily of dermatan and chondroitin sulfate, are attached to long core proteins. In AC, ACAN is more strongly expressed than COL2A1, another ECM component of cartilage (Chambers et al. 2002). The C terminus of ACAN binds to several ECM proteins, including tenascin and fibrin (Aspberg 2012). ACAN interacts with cell surface receptors and plays important roles in cell–ECM crosstalk, signaling factor storage and release, and cell division and migration (Cortes et al. 2009; Mouw et al. 2014). Hyaluronan in ACAN aggregates interacts with CD44 and syndecan receptors expressed on the surface of chondrocytes and regulates cartilage homeostasis (Bishop et al. 2007; Knudson et al. 2000). Hyaluronan in ACAN also binds to many ECM proteins, such as tenascin, fibers, and fibrillin, and regulates the functional stability of the matrix (Miura et al. 1999). Mutations in the human ACAN gene disrupt the Aggrecan–Tenascin interaction, resulting in a wide range of nonlethal skeletal dysplasia manifestations, including spondyloepiphyseal dysplasia, which is characterized by short stature and early OA (Gleghorn et al. 2005; Thompson et al. 2009), and various short stature syndromes with accelerated bone maturation (Nilsson et al. 2014; Gibson and Briggs 2016). In mice, homozygous frameshift mutations in ACAN result in perinatal lethality and a cartilage matrix deficiency phenotype characterized by cleft palate and dwarfism (Watanabe et al. 1994). *Agc1-IRES-creERT2* mice were generated and used for cartilage-specific gene knockout

Table 7.3 Summary of signaling pathway associated with articular cartilage formation

Name	Function	Down(dysregulation)	References
IHH	Regulates joint morphogenesis	Malformed phalanges and irregular joint shape	Storm and Kingsley (1999), Koyama et al. (2007), Später et al. (2006a), Minina et al. (2001), Mak et al. (2006, 2008), Wu et al. (2016), St-Jacques et al. (1999), Baur et al. (2000), Niedermaier et al. (2005), Koziel et al. (2005), Huang et al. (2016), Rockel et al. (2016)
BMP	It has chondrogenic activity and is negatively regulated by Noggin and chordin (BMP antagonist) in IZ to form joints	Significant impairment of skeletal formation	Koyama et al. (2008), Merino et al. (1999), Mak et al. (2006, 2008), Ray et al. (2015), Singh et al. (2018a, b), Seemann et al. (2005), Winslow and Burke (2010), Brunet et al. (1998), Wijgerde et al. (2005), Seo and Serra (2007), Garrison et al. (2017), Gelse et al. (2012), Chang et al. (2019a), Gamer et al. (2018)
TGF- β	Inhibitory effects on chondrogenesis in limb bud	Impairment of limb development, including arthroplasty	Wu et al. (2016), Mak et al. (2006), Seo and Serra (2007), Miyatake et al. (2016), Chavez et al. (2017, 2019), Pelton et al. (1991), Spagnoli et al. (2007), Bénazet et al. (2012), Lim et al. (2015), Guo et al. (2004), Knickmeyer et al. (2018), Aykul and Martinez-hackert (2016), Wakefield and Hill (2013), Oshimori and Fuchs (2012), Lyons and Rosen (2019), Zhang et al. (2005)
FGF18	Involved in joint formation by affecting Ihh/PTHrP and canonical Wnt signaling	Impaired skeletal development	Rockel et al. (2016), Itoh and Ornitz (2008), Ohbayashi et al. (2002), Liu et al. (2002), Shiang et al. (1994), Chen et al. (1999, 2001), Naski et al. (1998), Minina et al. (2002), Li et al. (2010), Reinhold and Naski

(continued)

Table 7.3 (continued)

Name	Function	Down(dysregulation)	References
			(2007), Salva and Merrill (2017)
PTHrP	Induces lubricin and contributes to AC development and homeostasis	Endochondral ossification disorder	Ogawa et al. (2014), Karaplis et al. (1994), Weir et al. (1996), Lanske et al. (1996), Guo et al. (2009), MacIca et al. (2011), Chen et al. (2006, 2008)
EGFR	Important roles in endochondral ossification, chondrocyte differentiation, and growth plate formation	Decreased expression of Sox9 and <i>Prg4</i> and progressive age-related joint degeneration	Zhang et al. (2007b, 2011b), Jia et al. (2016), Qin and Beier (2019), Wei et al. (2021), Shepard et al. (2013), Pest et al. (2014), Bellini et al. (2020)

and functional analysis (Henry et al. 2009). Young mice showed no difference in the expression of *Acan* and *Col2a1*, whereas older mice expressed *Acan* but not *Col2a1* (Chambers et al. 2002). However, *Agc1^{Cre/Cre}* mice showed a 50% reduction in both *Acan* mRNA and protein levels, exhibiting changes in body weight and dwarfism (Rashid et al. 2017).

7.2.1.3 Lubricin (PRG4)

Lubricin, encoded by *PRG4*, is a proteoglycan specifically expressed on the joint surface and synovium that reduces joint friction owing to its lubricating properties (Jay et al. 2001). In humans, deletions or mutations in *PRG4* have been implicated in the development of camptodactyly–arthropathy coxa–pericarditis syndrome with congenital strabismus and noninflammatory arthritis that develops in childhood (Marcelino et al. 1999), but no specific joint abnormalities are observed immediately after birth (Bahabri et al. 1998). Mechanical loading and PTHrP, TGF- β , and Wnt signaling are upstream regulators of *Prg4* (Koyama et al. 2008; Niikura and Reddi 2007; Ogawa et al. 2014; Matsuzaki et al. 2018). In *Prg4* knockout mice, there is no change in skeletal formation, but the AC surface gradually becomes abnormal with aging (Rhee et al. 2005; Coles et al. 2010; Hill et al. 2015), indicating that *PRG4* contributes significantly to AC homeostasis over time. In analyses of mouse embryos, *Prg4* mRNA was detected at E15.5 (Rhee et al. 2005). *Prg4*-positive cells present in the cartilage surface layer at E17.5 are found on the articular surface even after birth and spread to the deeper layers of AC with aging. *Prg4*-positive cells present on the articular surface of the embryo serve as the progenitor cell population for deeper layers of the mature AC (Kozhemyakina et al. 2015). Thus, *Prg4*-positive cells in the superficial zone are thought to be cartilage stem/progenitor cells that develop into articular chondrocytes (Kozhemyakina et al. 2015; Li et al. 2017), and factors that affect *Prg4* expression may have a significant impact on AC development. Signaling factors such as TGF β R2 (Li et al. 2013; Miyatake et al. 2016; Chavez et al. 2017, 2019) and MIG6 (Zhang et al. 2007b, 2011b; Jia et al. 2016; Qin

Table 7.4 Summary of transcription factors associated with articular cartilage formation

Name	KO mouse phenotype	Effect for cartilage	Downstream genes	Function	References
SOX9	Mesoderm condensation dysplasia	Protective	Col2a1, Aggrecan, Col10a1, Runx2	Essential transcription factor in chondrocyte differentiation	Bi et al. (1999), Foster et al. (1994), Wagner et al. (1994), Akiyama et al. (2002), Bell et al. (1997), Lefebvre et al. (1997), Ng et al. (1997), Zhou et al. (1998), Haseeb et al. (2021), Bagheri-Fam et al. (2006), Leipoldt et al. (2007), Fonseca et al. (2013), Benko et al. (2009), Mead et al. (2013), Yao et al. (2015), Mochizuki et al. (2018), Hall et al. (2017), Li et al. (2004), Yang et al. (2011), Park et al. (2011), Kim et al. (2011), Im et al. (2011), Garza-Veloz et al. (2013), Lu et al. (2015), Cao et al. (2018), Zhang et al. (2017a, 2021b), Song and Park (2020)
FOXO1, 3, 4	OA-like pathology in the natural course with decreased expression of PRG4	Protective	Prg4	Protect against cellular and organismal aging	Akasaki et al. (2014), Matsuzaki et al. (2018), Eelen et al. (2016), Duffy et al. (2020), Wang et al. (2020a), Brunet et al.

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Table 7.4 (continued)

Name	KO mouse phenotype	Effect for cartilage	Downstream genes	Function	References
					(1999), Kops et al. (1999), Biggs III et al. (1999), Tran et al. (2002), Paik et al. (2007), Jacobs et al. (2003), Burgering (2003), Horst and Burgering (2007), Eijkelenboom and Burgering (2013)
NFATc	Embryonic lethal E13.5 due to defective cardiac valves	Protective	Sox9, Prg4	Positive regulation of Sox9 and Prg4	Greenblatt et al. (2010, 2013), Wang et al. (2009), Ranger et al. (1998), Sitara and Aliprantis (2010), Koga et al. (2005)
CREB	Dysplasia of proliferating zone and hypertrophic zone of growth plate	Protective	Prg4	Promotion of chondrocyte proliferation via Ptch	Ogawa et al. (2014), Zhang et al. (2021a)
HIF1 α	Mesoderm condensation dysplasia	Protective	Sox9, Vegf, NF-k β signaling	Negative regulation for Mmp13 and Hif-2 α through suppression of NF-k β signaling	Bracken et al. (2003), Semenza (2000), Lando et al. (2002), Schofield and Ratcliffe (2004), Okada et al. (2020)
HIF2 α	Resistance to osteoarthritis development	Offensive	COL10a1, MMP13, VEGFA	Induction of hypertrophic change and inflammatory degenerative changes of articular chondrocytes	Saito et al. (2010), Stewart et al. (2006), Yang et al. (2010), Amarilio et al. (2007), Ito et al. (2021)
YBX1		Protective	p54nrb, Col2a1	Positive regulation of	Schmid et al. (2013), Troiano

(continued)

Table 7.4 (continued)

Name	KO mouse phenotype	Effect for cartilage	Downstream genes	Function	References
				Col2a1 by activating p54nrb transcription	et al. (2015), Faber et al. (2009), Zhang et al. (2022a), Kwon et al. (2018)
SOX5,6	Single null mice: mild phenotype, double null mice: cartilage dysplasia	Protective	Col2a1, Aggrecan	Enhance the DNA-binding capacity and transcriptional activity of SOX9	Bell et al. (1997), Lefebvre et al. (1997), Ng et al. (1997), Zhou et al. (1998), Yamashita et al. (2012)

and Beier 2019; Wei et al. 2021; Shepard et al. 2013; Pest et al. 2014; Bellini et al. 2020), transcription factors such as FOXO (Akasaki et al. 2014; Matsuzaki et al. 2018; Eelen et al. 2016; Duffy et al. 2020; Wang et al. 2020a), NFATc (Greenblatt et al. 2013; Wang et al. 2009), CREB (Ogawa et al. 2014; Zhang et al. 2021a), and arginine methyltransferase PRMT5 (Bedford and Clarke 2009; Tee et al. 2010; Norrie et al. 2016; Ramachandran et al. 2019) have been reported as regulators of PRG4 expression.

7.2.1.4 Tenascin-C (TN-C)

TN-C is a glycoprotein component of the ECM. In addition to TN-C, there are three other tenascin family members, namely tenascin-W, tenascin-X, and tenascin-R. TN-C consists of four domains, including a Tenascin assembly domain, epidermal growth factor-like repeat sequence, fibronectin type III-like repeat sequence, and fibrinogen-like globulin domain (Hasegawa et al. 2018, 2020). TN-C forms mechanical and biochemical signals within the cellular microenvironments of various tissues by regulating cell adhesion (Midwood et al. 2016). Moreover, it is expressed in the IZ and is involved in chondrocyte differentiation during embryonic cartilage maturation. Cartilage-specific ECM proteins are produced before TN-C expression is lost (Pacifici et al. 1993; Pacifici 1995). Subsequently, by 4 weeks of age, TN-C expression was observed in the perichondrium (Pacifici 1995; Edwall-Arvidsson and Wroblewski 1996) and remained expressed in the AC but not in the growth plates. Its expression was markedly reduced during chondrocyte maturation and was nearly absent in adult AC (Pfander et al. 2004). TN-C contributes to musculoskeletal remodeling (Järvinen et al. 1999; Flück et al. 2008), and the acquisition of load-dependent regenerative functions of joints after birth (Namba et al. 1998; Brommer et al. 2005). Recently, TN-C knockout mice exhibited reduced production of chondrocyte organelles at 2 months of age, indicating the possible involvement of TN-C

in postnatal articular chondrocyte differentiation and maturation (Gruber et al. 2020).

7.2.2 Signaling-Related Proteins

7.2.2.1 Transforming Growth Factor- β s (TGF- β s)

TGF- β ligands bind to TGF- β type II receptors (Tgfr2) and recruit TGF- β type I receptors (Tgfr1) to activate intercellular signaling cascades such as Smad2/3. TGF- β signaling is important for joint development (Wu et al. 2016; Pelton et al. 1991). *Tgfr2*-positive cells are expressed in the IZ from E12.5 to E16.5 in mice (Spagnoli et al. 2007). *Tgfr2* knockout in the early mesenchyme using *Prx1-cre* results in *Gdf5*-positive cells that cannot migrate to the IZ and fail to form IZs in the phalanges (Seo and Serra 2007; Spagnoli et al. 2007), leading to severe impairment of limb development, including joint formation (Bénazet et al. 2012; Lim et al. 2015). Furthermore, TGF- β and Wnt/ β -catenin signaling exerts inhibitory effects on chondrogenesis in limb bud cultures (Mak et al. 2006; Seo and Serra 2007; Guo et al. 2004). Inhibition of chondrogenesis in the precartilaginous tissue is necessary for IZ generation, and BMP signaling must be suppressed. The mechanisms of inhibition of BMP pathway by TGF- β involve various mechanisms, including the induction of BMP antagonists (Knickmeyer et al. 2018), competition for type II receptors shared by TGF- β pathway ligands and BMP ligands (Aykul and Martinez-hackert 2016), competition for Smad4 (Wakefield and Hill 2013), and induction of genes that limit BMP signaling (Oshimori and Fuchs 2012; Lyons and Rosen 2019). Smad4 deficiency in early mesenchymal lineages mainly affects endochondral ossification in chondrocytes, with a less significant effect on joints (Zhang et al. 2005).

Tgfr2 expression is maintained in joints and surrounding tissues throughout adulthood, apart from transient expression in the IZ (Li et al. 2013). TGF- β signaling also induces *Prg4* expression via the Smad3-mediated signaling pathway (Miyatake et al. 2016; Chavez et al. 2017). Reduced expression of the TGF β 2 has been associated with OA in clinical samples, and mice with a dominant-negative mutation (DNIIR) in *Tgfr2* exhibit OA. In DNIIR mice, *Prg4* expression in the superficial zone of the AC is significantly reduced at 2 months of age (Chavez et al. 2019).

7.2.2.2 EGFR Signaling

Mitogen-inducible gene 6 (*Mig6*) is an inhibitor of EGFR feedback (Zhang et al. 2007b). *Mig6* knockout activates EGFR signaling, which plays an important role in endochondral ossification, chondrocyte differentiation, and growth plate formation (Zhang et al. 2011b). *Mig6* is involved in the regulation of *Prg4* expression in the AC tissue and has a protective function against joint destruction (Jia et al. 2016; Qin and Beier 2019; Wei et al. 2021). *Mig6* conditional knockout mice with *Col2a1-cre* or *Prx1-cre* showed activation of EGFR signaling and increased knee joint thickness (Shepard et al. 2013; Pest et al. 2014). Mice overexpressing heparin-binding EGF-like growth factor, a ligand for EGFR, using the *Col2-cre* strain had thickened AC owing to an expanded pool of chondroprogenitors with enhanced proliferation

ability, survival rate, and lubricant production (Wei et al. 2021). In contrast, mice overexpressing *Mig6* with the *Col2-cre* strain showed no abnormalities in AC development; however, the reduced EGFR activity decreased *Sox9* and *Prg4* expression in the AC, resulting in progressive joint degeneration with aging (Bellini et al. 2020). Thus, downstream signaling of EGFR is involved in the induction of *Prg4* expression.

7.2.2.3 Fibroblast Growth Factor 18 (FGF18)

FGF18 belongs to the FGF family and is involved in AC development; *Fgf18*-deficient mice die in the neonatal period and show impaired skeletal development (Itoh and Ornitz 2008; Ohbayashi et al. 2002; Liu et al. 2002). Mutations in *FGFR3*, the receptor for FGF18, are known to cause severe dwarfism and chondrodysplasia in mice and humans (Shiang et al. 1994; Chen et al. 1999, 2001), and FGF18/*FGFR3* signaling is essential for skeletal development. Although FGF18 is expressed in the IZ cells (Ohbayashi et al. 2002; Liu et al. 2002), *Fgf18* knockout does not affect the formation of most limb joints; hence, its role in skeletal development remains unclear (Itoh and Ornitz 2008; Ohbayashi et al. 2002; Liu et al. 2002; Shiang et al. 1994; Chen et al. 1999). FGF18/*FGFR3* signaling is closely associated with IHH-PTHrP and canonical Wnt signaling, and the loss of *FGFR3* signaling decreases *Ihh* ligand and *Pthrp* receptor expression (Naski et al. 1998; Minina et al. 2002; Li et al. 2010). Conversely, activation of IHH signaling in the joint space induced by loss of *Patched 1* expression and expression of *SmoM2* inhibits AC and meniscus formation by inhibiting Wnt/ β -catenin target genes, such as *Fgf18*, thus leading to ectopic chondrogenesis (Rockel et al. 2016). Furthermore, the phenotype induced by IHH is abolished by β -catenin stabilization or FGF18 (Rockel et al. 2016). *Fgf18* is a direct transcriptional target of canonical Wnt signaling (Reinhold and Naski 2007). These results indicate that FGF18 is involved in joint formation by interactively influencing *Ihh*/PTHrP and canonical Wnt signaling (Salva and Merrill 2017).

7.2.2.4 Parathyroid Hormone-Related Peptide (PTHrP)

Genetic modification in PTHrP causes impaired endochondral ossification but no major joint abnormalities (Karaplis et al. 1994; Weir et al. 1996; Lanske et al. 1996; Guo et al. 2009; MacIca et al. 2011). PTHrP-expressing cells remain in the AC throughout life after the loss of IHH signaling (MacIca et al. 2011; Chen et al. 2006, 2008). PTH/PTHrP signaling induces lubricin expression (Ogawa et al. 2014). These findings suggest that PTHrP contributes to AC development and homeostasis during the postnatal period.

7.2.2.5 CD44

CD44 is the major cell surface receptor for hyaluronic acid (HA). It is a transmembrane protein containing an N-terminal extracellular domain that binds HA and a short cytoplasmic domain associated with the cytoskeleton. In the inner IZ layer, the hyaluronan-binding proteins hyaluronan synthase and CD44 are expressed, and the activity of uridine diphosphoglucose dehydrogenase (UDPGD), an enzyme involved

in sugar chain synthesis, is increased (Pitsillides et al. 1995; Dowthwaite et al. 1998). Knockout of hyaluronan synthase 2 (Has2), an enzyme involved in the synthesis of hyaluronan, in mice demonstrated defective joint cavity formation (Matsumoto et al. 2009). These findings suggest that HA synthesis and accumulation are involved in joint cavity formation. Additionally, HA functions as a regulator or co-activator of the BMP signaling network in articular chondrocytes (Peterson et al. 2004), suggesting that the interaction between HA and CD44 may be involved in chondrocyte maturation.

Sub-clustering analysis of IZ-forming cell clusters showed that CD44 was expressed in clusters with high expression of cartilage-related and tendon-related genes, *Gdf5*, *Tppp3*, and *Sox9*. In addition, this cluster also highly expresses *Igfbp2*, which is specifically expressed along the surface of the joint cavity (Pazin et al. 2014). CD44 is specifically expressed on the articular surface (Bian et al. 2020).

7.3 Transcription Factors

Transcription factors are proteins involved in the initiation and regulation of gene transcription (Fig. 7.1). They usually bind to promoter regions and regulate the expression of downstream genes. During development, transcription factors coordinate the expression of multiple genes and promote the differentiation of stem cells to mature cells. As mentioned previously, early AC development shares a common mechanism with endochondral ossification, and several key transcription factors are shared among the two processes (Long and Ornitz 2013; Nakamichi et al. 2020). In this section, we review the functions of some of the transcription factors that have been well researched in AC development and homeostasis.

7.3.1 SRY-Box9 (SOX9)

SOX9 is an essential transcription factor in chondrocyte differentiation (Bi et al. 1999; Foster et al. 1994; Wagner et al. 1994). Sox9-knockout cells do not aggregate undifferentiated mesenchymal stem cells or initiate chondrogenesis (Bi et al. 1999; Akiyama et al. 2002), suggesting its involvement undifferentiated stem cell aggregation and their differentiation into chondrogenic progenitor cells. As a member of the SOX-trio, SOX9 regulates SOX5 and SOX6 activity and *Col2a1* and *aggrecan* expression (Bell et al. 1997; Lefebvre et al. 1997; Ng et al. 1997; Zhou et al. 1998). SOX9 expression is maintained in mature chondrocytes, suggesting that SOX9 sustains cartilage homeostasis during the developmental phase (Haseeb et al. 2021). This finding is also supported by the observation that the knee joints of *Acan-creERT2: Sox9^{flox/flox}* mice gradually degenerate with age (Haseeb et al. 2021).

Several studies have been conducted on *SOX9* enhancers. In studies focusing on campomelic dysplasia and acampomelic campomelic dysplasia, *SOX9* enhancers have been identified within a noncoding region of approximately 2 MB, located

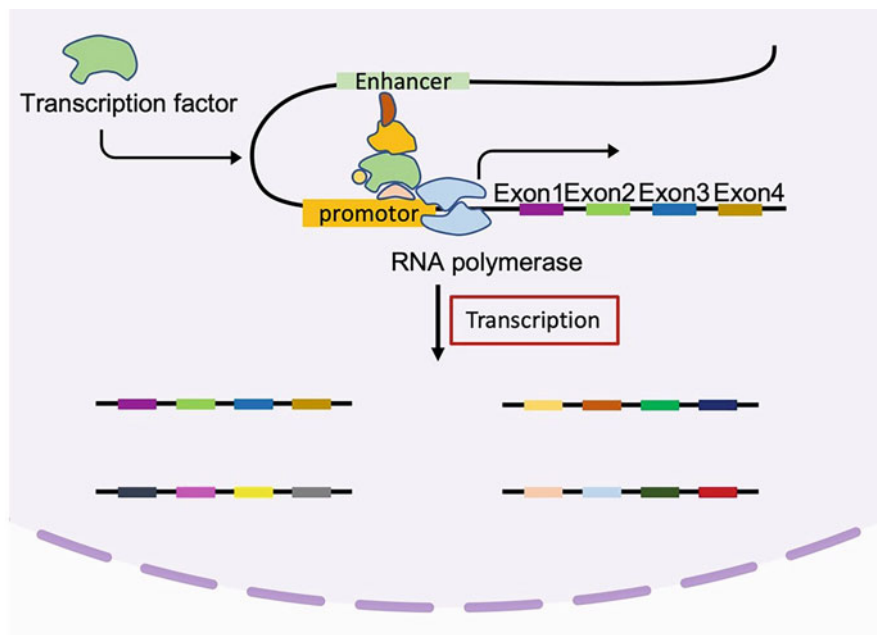


Fig. 7.1 Schematic overview of the function of transcription factors

between *SOX9* and the coding gene *KCNJ2*. These enhancers are responsible for activating transcription in several organs, including the cartilage (Bagheri-Fam et al. 2006; Leipoldt et al. 2007; Fonseca et al. 2013; Benko et al. 2009; Mead et al. 2013; Yao et al. 2015). Furthermore, an enhancer exists approximately 1 Mb upstream of *SOX9* in costal chondrocytes and the transcription factor STAT3 regulates *SOX9* expression through this region, suggesting STAT3 is an important modulator of cartilage development (Mochizuki et al. 2018; Hall et al. 2017).

Many studies have been performed to evaluate the potential of targeting *SOX9* as a therapeutic tool (Li et al. 2004; Yang et al. 2011; Park et al. 2011; Kim et al. 2011; Im et al. 2011; Garza-Veloz et al. 2013; Lu et al. 2015; Cao et al. 2018; Zhang et al. 2017a, 2021b) and all of them reported a certain level of efficacy. However, a more efficient delivery system and developments in tissue engineering are expected to improve its suitability for clinical application (Song and Park 2020).

7.3.2 Forkhead Box Class O (FOXO)

The FOXO family, consisting of FOXO1, FOXO3, FOXO4, and FOXO6, comprises evolutionarily conserved transcription factors with significant effects on development, longevity, and aging (Brunet et al. 1999; Kops et al. 1999; Biggs III et al. 1999; Tran et al. 2002). FOXO1, FOXO3, and FOXO4 are ubiquitously expressed and show high functional overlap (Paik et al. 2007), whereas FOXO6 is mainly

localized in the brain (Jacobs et al. 2003). FOXO is a downstream target of the protein kinase Akt, and the PI3K-Akt-FOXO signaling pathway influences multiple cellular functions, including cell growth and survival (Burgering 2003; Horst and Burgering 2007). FOXO responds to environmental stimuli and regulates the dynamic gene expression programs involved in physiological and pathological processes (Eijkelenboom and Burgering 2013).

In cartilage tissue, FOXO expression decreases with age (Akasaki et al. 2014). Decreased FOXO expression has also been observed in human and mouse OA cartilage. In mice, induction of triple knockout of *FOXO 1, 3, and 4* in adulthood leads to OA-like pathology in the natural course, notably characterized by decreased PRG4 expression (Matsuzaki et al. 2018). In vitro, overexpression of *FoxO* in chondrocytes promotes cell survival and anabolic responses to cellular stress conditions (Matsuzaki et al. 2018; Eelen et al. 2016). FOXO also binds to gene regions encoding extracellular organelles in chondrocytes (Duffy et al. 2020). These findings suggest that FOXO plays an essential role in AC maintenance. In fact, OA induction in mice overexpressing *FoxO* in adulthood inhibited OA progression (Wang et al. 2020a). Furthermore, TGF β , which has important functions in AC development, is also known to induce FOXO expression (Akasaki et al. 2014; Wang et al. 2020a), and OA changes caused by the deletion of tissue-specific TGF β signaling are similarly rescued by tissue-specific *FoxO1* overexpression in mouse models (Wang et al. 2020a).

7.3.3 Nuclear Factor of Activated T Cells (NFATc)

The NFAT family of transcription factors plays diverse roles in a wide range of tissues, from heart valve and cerebrovascular development to innate and adaptive immune responses (Ranger et al. 1998; Sitara and Aliprantis 2010; Greenblatt et al. 2010). In osteoblasts, NFAT transcription factors cooperate with osterix to promote bone formation (Koga et al. 2005). Moreover, NFATc plays an important role in AC formation and Prg4 expression. *Nfatc1* is strongly expressed in superficial regions of the AC (Greenblatt et al. 2013). *Nfatc1* knockout mice are embryonically lethal at E13.5 due to defective cardiac valves (Ranger et al. 1998). No obvious morphological abnormalities in the AC were observed in *Nfatc1* cKO mice using the *Col2-cre* strain (Greenblatt et al. 2013). Conversely, in *Nfatc2* KO mice, there is an imbalance between cartilage matrix degradation and synthesis, and *Col2a1* and *Acan* expression in the femoral head of 3-month-old mice is reduced, showing OA-like symptoms (Wang et al. 2009). *Col2-cre:Nfatc1^{fllox/flox}*, *Nfatc2^{-/-}* mice show reduced *Sox9* and *Prg4* expression in the AC and develop severe OA (Greenblatt et al. 2013).

7.3.4 cAMP Response Element-Binding Protein (CREB)

CREB binds to the cAMP response element and regulates transcription (Montminy and Bilezikjian 1987). Recent studies that performed RNA sequencing of superficial

and deep AC cells confirmed that CREB5 is specifically expressed in the superficial layers (Zhang et al. 2021a) and that *Creb5* overexpression in deep chondrocytes strongly induces *Prg4* expression (Zhang et al. 2021a). Furthermore, mechanical stress induces CREB phosphorylation and increases *Prg4* expression (Ogawa et al. 2014). These findings suggest that CREB5 plays an important role in regulating *Prg4* expression.

7.3.5 Hypoxia-Inducible Factor (HIF) 1 α and 2 α

HIF proteins belong to the basic helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) transcription factor family, which consists of α - and β -subunit members (Bracken et al. 2003; Semenza 2000). Under normal oxygen concentrations, α -subunit members undergo oxygen-dependent hydroxylation, are ubiquitinated, and degraded by the proteasome (Lando et al. 2002; Schofield and Ratcliffe 2004). In contrast, under hypoxic conditions, it is neither hydroxylated nor degraded and forms heterodimers with constitutive β -subunit members. This heterodimer activates transcription of the target gene by binding to a consensus sequence called a hypoxia-responsive element in the promoter (Semenza 2000).

In a recent study, HIF-1 α expression was gradually decreased in the AC of OA-induced mouse models, leading to functional analysis of AC (Okada et al. 2020). The results showed that HIF-1 α negatively regulates *Mmp13* and *Hif-2 α* by suppressing NF- κ B signaling, suggesting that maintaining HIF-1 α expression may be important for the overall maintenance of AC homeostasis (Okada et al. 2020).

HIF-2 α is highly expressed in differentiated chondrocytes and functions independently of oxygen-dependent hydroxylation (Saito et al. 2010; Stewart et al. 2006; Yang et al. 2010). HIF-2 α expression is higher in osteoarthritic cartilage than in normal AC. *Epas1* (HIF-2 α is encoded by *EPAS1*)-heterozygous-deficient mice exhibit resistance to OA development. HIF-2 α positively regulates COL10A1, MMP13, and VEGFA expression and is assumed to be involved in the induction of hypertrophic changes and inflammatory degenerative changes in the articular chondrocytes (Saito et al. 2010; Yang et al. 2010; Amarilio et al. 2007). Therefore, HIF-2 α has been recognized as a target to be inhibited to maintain AC homeostasis, and the mechanism of its suppression and regulation by miRNAs has been investigated and is described in Sect. 7.4.2 (Ito et al. 2021).

7.3.6 Y-Box Binding Protein 1 (YBX1)

YBX1 regulates *Col2a1* expression by activating p54nrb transcription in an MIA/CD-RAP-dependent manner and is known to be a transcription factor involved in chondrogenic differentiation (Schmid et al. 2013). However, YBX1 is also known to activate the P13K/AKT signaling pathway, and YBP1-induced chondrocytes show increased PI3K, p-PI3K, AKT, and p-AKT expression, leading to the inhibition of chondrocyte proliferation and induction of cell death (Troiano et al. 2015;

Faber et al. 2009). YBX1 is upregulated in OA AC compared to that in normal AC, indicating that these negative homeostasis mechanisms may be involved in OA (Zhang et al. 2022a). YBX1 negatively regulates cellular senescence through its involvement in post-transcriptional translation as an RNA-binding protein in epidermal progenitor cells (Kwon et al. 2018); the same function may exist in AC.

7.4 Biomolecules Affecting Cartilage Homeostasis

Maintenance of AC homeostasis demands the maintenance of the ability of the AC to produce ECM and inhibit structural destruction. Thus, it is necessary to understand and apply both direct and indirect mechanisms that enhance and maintain the ability of articular chondrocytes to produce ECM. The most well-known of the direct mechanism is the transcriptional regulatory mechanism that regulates ECM expression at the mRNA level. Conversely, the indirect mechanism is a post-transcriptional regulatory mechanism that regulates mRNA degradation and suppresses cellular damage, and senescence.

7.4.1 RNA-Binding Proteins

RNA-binding protein (RBP) is a general term for proteins that bind to single- or double-stranded RNA present in cells and is a component of the ribonucleoprotein complex (Glisovic et al. 2008). RBPs are localized in the cytoplasm or nucleus and exist in the nucleus as complexes of proteins (heteroribonucleoproteins; hnRNPs) and immature precursor mRNAs (pre-mRNAs) during the maturation and export of mRNA (Glisovic et al. 2008). RBPs play important roles in a variety of cellular functions, and many have been reported to play key roles in post-transcriptional regulatory mechanisms such as RNA splicing, polyadenylation, mRNA stabilization, localization, and translation (Glisovic et al. 2008) (Fig. 7.2). Approximately 1542 RBPs constitute 7.5% of all protein-coding genes in the human genome (Gerstberger et al. 2014). This diversity of RBP is thought to be closely related to the evolutionary increase in intronic sequences and noncoding RNAs and the mechanisms that regulate their expression. The study of RBPs has also been conducted in AC studies (Table 7.5).

7.4.1.1 Pre-transcriptional Regulation

54-kDa Nuclear RNA-Binding Protein (p54nrb)

p54nrb is an RBP involved in the transcriptional regulation of *Col2a1*, as identified by *Col2a1* promoter activity screening using ATDC5 cells (Hata et al. 2008). p54nrb is involved in pre-transcriptional regulatory mechanisms and interacts with SOX9 and promotes *Col2a1* transcription in an SOX9-dependent manner. Furthermore, the RNA recognition motif (RRM) of p54nrb is involved in *Col2a1* mRNA splicing and maturation (Hata et al. 2008). ATDC5 mutating the RRM of p54nrb results in

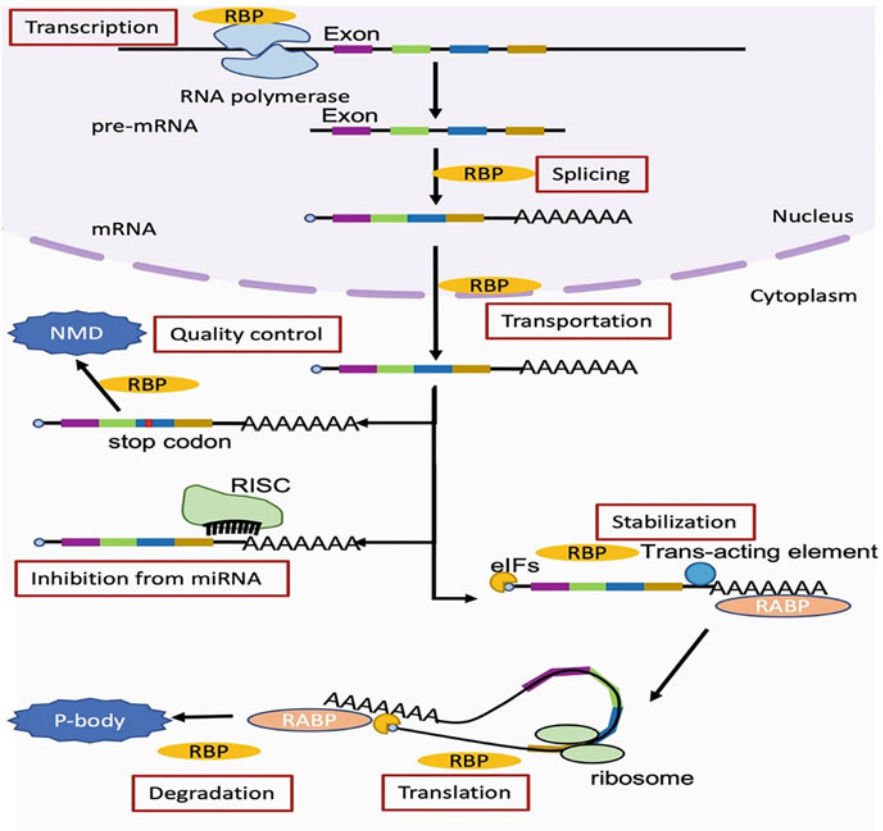


Fig. 7.2 Schematic overview of the function of RNA-binding protein. *RBP* RNA-binding protein, *NMD* nonsense-mediated mRNA decay

reduced transcriptional activity of *Col2a1*, and mutant p54nrh transgenic mice exhibit impaired endochondral ossification (Hata et al. 2008). p54nrh is transcriptionally regulated by melanoma inhibitory activity/cartilage-derived retinoic acid-sensitive protein (MIA/CD-RAP), which is expressed during chondrogenesis (Dietz and Sandell 1996; Schubert et al. 2010). MIA/CD-RAP-knockout mice show decreased p54nrh expression, resulting in impaired endochondral ossification (Schmid et al. 2010). However, AC formation in MIA/CD-RAP knockout mice is normal (Moser et al. 2002), and in a traumatic OA-induced mouse model, AC regeneration proceeds with proliferating chondrocytes (Schmid et al. 2010).

Table 7.5 Summary of RNA-binding protein associated with articular cartilage homeostasis

Gene name	Function	Effect for cartilage	Target genes	References
p54nrb	Antetranscriptional regulation	Protective?	Promotion of transcription of <i>COL2A1</i>	Hata et al. (2008), Dietz and Sandell (1996), Schubert et al. (2010), Schmid et al. (2010), Moser et al. (2002)
GNL3	Splicing	Offensive	Increment of the expression of IL-24, PTN	Gee et al. (2014), Zhu et al. (2021a)
FUS	Splicing	Protective	Stabilization of circRNA_PDE4B, circRNA_SLC7A2, and <i>SOX9</i>	Gu et al. (2014), Shen et al. (2021), Ni et al. (2021), Bai et al. (2020)
METLL3	Stability of mRNA	Offensive	Stabilization of <i>BCL2</i> , unstabilization of <i>SOX9</i>	Zhao et al. (2020), He et al. (2022a), Xiao et al. (2022), Chen et al. (2022)
TTP	Stability of mRNA	Offensive	Unstabilization of <i>SOX9</i> , <i>HSPA1A</i>	Bermudez et al. (2011), Sanduja et al. (2011), McDermott et al. (2016), Son et al. (2019)
HuR	Stability of mRNA	Protective	Unstabilization of <i>MMP13</i> , <i>COX2</i>	McDermott et al. (2016), Chen et al. (2020a)
SND1	Stability of mRNA	Offensive	Unstabilization of <i>HSPA5</i>	Lv et al. (2022)
TIA-1	Stress granule assembly	Protective	Inhibition of translation of <i>COX2</i>	Ansari and Haqqi (2016)
TDP-43	Stress granule assembly	Protective	Induction of SG formation	Huang et al. (2017, 2019a, b), Chang et al. (2021)
CPEB1	Regulation of translocation	Offensive	Extension of PolyA tail	Udagawa et al. (2013), Li et al. (2019), Chen et al. (2020a)
PUM1	Regulation of translocation	Protective	Inhibition of translation of <i>TLR4</i>	Yoon et al. (2022)

7.4.1.2 Splicing

Nucleolar GTP-Binding Protein 3 (GNL3)

GNL3 is an RBP identified by genome-wide association scans as a susceptibility gene allele associated with OA (Gee et al. 2014) that is also involved in splicing (Gee et al. 2014). *Cis*-acting regulatory polymorphisms in GNL3 and SPCS1 are present in OA (Gee et al. 2014). GNL3 may be involved in the expression of IL-24 and PTN

in SW1353 cells (Zhu et al. 2021a). A more detailed functional analysis is expected in the future.

Fused in Sarcoma (FUS)

FUS was detected in studies using mouse hypertrophic cartilage-derived cells as a predicted protein that binds to the *cis*-enhancer of *Col10A1* and RUNX2 (Gu et al. 2014). Later, it was reported to promote splicing of circPDE4B in articular chondrocytes (Shen et al. 2021). circPDE4B acts as a scaffold for RIC8 guanine-nucleotide exchange factor A (RIC8A)-midline 1 binding, thereby reducing the RIC8A-dependent p38 mitogen-activated protein kinase signaling pathway (Shen et al. 2021). In fact, intra-articular administration of circPDE4B in traumatic OA-induced mouse models inhibits OA progression (Shen et al. 2021) and promoted circSLC7A2 splicing. CircSLC7A2 has a sponge function for miR-4498 and produces an anti-inflammatory effect by maintaining TIMP3 expression (Ni et al. 2021). FUS is also involved in the maintenance of mRNA stability and, together with LncRNAMM2P, binds to *SOX9* mRNA to stabilize it and induce *SOX9* expression (Bai et al. 2020).

7.4.1.3 mRNA Stability

Methyltransferase 3 (METTL3)

METTL3, a m6A methyltransferase, promotes tumorigenesis by enhancing c-Myc mRNA stability through YTHDF1-mediated m6A modification (Zhao et al. 2020). In the cartilage field, METTL3-YTHDF1-mediated m6A modification inhibits chondrocyte apoptosis and autophagy during inflammation via Bcl2 mRNA stability (He et al. 2022a). It is also known to inhibit the induction of *Col2a1* expression by inducing *Sox9* mRNA methylation, making it unstable in the endplate cartilage (Xiao et al. 2022). In synovial cells, METTL3 impairs autophagy and promotes cellular senescence by modifying ATG7 m6A and decreasing mRNA stability. In a traumatic OA-induced model, this pathway was activated in synovial cells, thus accelerating OA; conversely, siMETTL3 modulates autophagy and suppresses OA progression (Chen et al. 2022).

Tristetraprolin (TTP)

TTP, also known as ZFP36 and TPA-inducible sequence 11 (TIS11), is an RBP. The ZFP36 family includes ZFP36L1, ZFP36L2, and ZFP36L3, all of which have two ZF motifs (CCCH) that bind to an AU-rich element (ARE) in the 3'-UTR of the target mRNA, resulting in mRNA destabilization and degradation (Bermudez et al. 2011; Sanduja et al. 2011). In chondrocytes, TTP binds to the ARE of the 3'-UTR of *SOX9* mRNA and acts as a suppressor of *SOX9* (McDermott et al. 2016). ZFP36L1, a member of the ZFP36 family, is also upregulated in OA chondrocytes and cartilage in traumatic OA-induced mouse models. ZFP36L1 silencing suppresses OA progression in these models because ZFP36L1 targets HSPA1A and 1 B, heat-shock protein 70 (HSP70), which has protective effects against inflammation, oxidative stress, and cell death (Son et al. 2019). In fact, HSPA1A induction in the knee joint

of a traumatic OA-induced model suppressed OA progression (Son et al. 2019). These findings indicate that TTP exerts a destructive effect on AC.

Human Antigen R (HuR)

HuR is an RBP with three RRM domains that binds to the ARE of target mRNA and is involved in mRNA stabilization. In chondrocytes, HuR targets MMP13 and destabilizes it by binding to its mRNA (McDermott et al. 2016). HuR also targets COX2, inducing the production of pro-inflammatory prostanooids and potentially destabilizing the mRNA (Chen et al. 2020a). These results suggest that HuR has a protective effect against AC.

Staphylococcal Nuclease and Tudor Domain-Containing 1 (SND1)

SND1 promotes GPX4 degradation by suppressing HSPA5 expression in chondrocytes through HSPA5 mRNA destabilization, thereby promoting ferroptosis in OA chondrocytes (Lv et al. 2022). Thus, SND1 is predicted to have destructive effects on the AC.

7.4.1.4 Stress Granule Assembly

T-Cell-Restricted Intracellular Antigen 1 (TIA-1)

TIA-1 is an RBP with three RRM domains and is involved in stress granule (SG) assembly. SGs formed by TIA-1 sequester COX2 mRNA and inhibit its translation under experimental inflammatory stimuli in chondrocytes (Ansari and Haqqi 2016).

Transactive Response DNA-Binding Protein 43 kDa (TDP-43)

TDP-43 is an RBP involved in SG assembly, characterized by two RRM domains, a nuclear localization signal (NLS), a nuclear export signal, and a Gly rich domain. TDP-43 induces RACK1 and G3BP1 expression to promote SG formation, which has chondrocyte anti-inflammatory, anti-cell death, and anti-angiogenic activity (Huang et al. 2017, 2019a, b). TDP-43 expression was reduced in traumatic OA-induced mouse models, and TDP-43 induction into the knee joint can inhibit OA progression (Chang et al. 2021).

7.4.1.5 Regulation of Translocation

Cytoplasmic Polyadenylation Element-Binding Protein 1 (CPEB1)

CPEB1 is an RBP that binds to the 3'-UTR-cytoplasmic polyadenylation element of target mRNAs and regulates mRNA translation by elongating or removing the poly (A) tail (Udagawa et al. 2013). CPEB1 is upregulated in OA cartilage (Li et al. 2019; Chen et al. 2020a). Induction of inflammation in chondrocytes induces CPEB1 expression and enhances catabolism; however, CPEB1 downregulation attenuates this enhancement, suggesting that CPEB1 may be involved in OA pathogenesis (Li et al. 2019).

Pumilio RNA-Binding Family Member 1 (PUM1)

PUM1 interacts with the 3'-UTR of Toll-like receptor 4 (TLR4) to repress TLR4 mRNA translation and modulate the activity of NF- κ B, a master regulator of the aging process in mesenchymal stem cells (Yoon et al. 2022). Similar processes work in chondrocytes, with PUM1 knockdown in human chondrocytes showing a reduction in cartilage markers and PUM1 overexpression suppressing inflammation-induced reduction of cartilage markers. Moreover, introducing PUM1 into the knee joints of traumatic OA-induced mouse models demonstrated a suppressive effect on OA progression in AC (Yoon et al. 2022).

7.4.2 MicroRNAs (miRNAs)

miRNAs are short, noncoding RNAs of approximately 22 bases that are highly conserved in many species and repress gene expression primarily by binding to the 3'-UTR of mRNAs (Lee et al. 1993; Wightman et al. 1993). miRNAs are deeply involved in various physiological phenomena and pathological conditions, including chondrocyte differentiation (Ambros 2004), and more than half of human genes may be regulated by miRNAs. They are mainly transcribed as primary miRNAs, cleaved in the nucleus and cytoplasm, and finally biosynthesized into mature miRNAs (Bartel 2018) (Fig. 7.3). Kobayashi et al. generated a *Prg4-creERT* mouse model in which the Droscha gene, which plays a central role in cytoplasmic cleavage, was knocked down in chondrocytes in a time-specific manner and this reduced the expression of mature miRNAs in chondrocytes (Kobayashi et al. 2015). In this model, apoptosis of articular chondrocytes was enhanced and ECM production was decreased, suggesting that the maintenance of miRNA expression plays an important role in AC homeostasis (Kobayashi et al. 2015). Several reports have described miRNAs that, due to their unique functions, play a role in suppressing the factors involved in AC degeneration (Table 7.6). In this section, we review representative miRNAs involved in AC homeostasis.

7.4.2.1 miRNA140

miRNA140 is the most highly expressed miRNA in cartilage and plays important roles in endochondral ossification and AC development and homeostasis (Wienholds et al. 2005; Tuddenham et al. 2006; Miyaki et al. 2009). miRNA140 is located in the intron region of WWP2, and the expression of miRNA140 and WWP2 is regulated by SOX9 (Nakamura et al. 2012; Yamashita et al. 2012). In this regulatory process, SOX5 and SOX6 also enhance the DNA-binding capacity and transcriptional activity of SOX9 (Yamashita et al. 2012). The AC of miR140 knockout mice is morphologically normal at 1 month of age but gradually changes to OA with age. Conversely, miRNA140-overexpressing mice are resistant to drug-induced OA (Miyaki et al. 2010). ADMATS5 is a target of miRNA140 in chondrocytes, and suppression of this target is thought to produce an inhibitory effect on OA development (Miyaki et al. 2010). Other known targets of miRNA140 in chondrocytes include DNPEP (Nakamura et al. 2011), RALA (Karlsen et al.

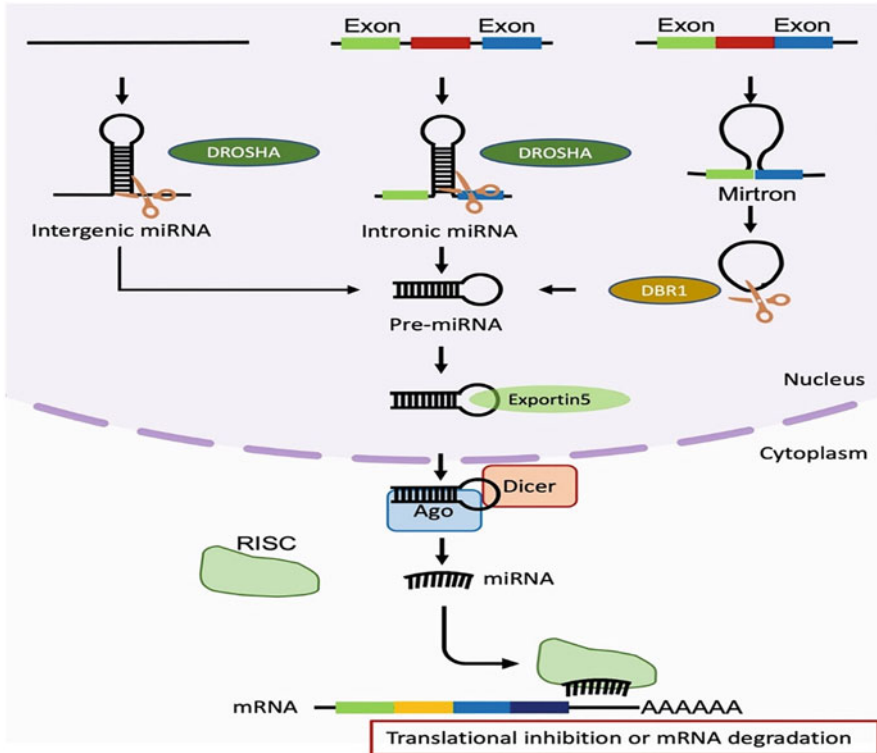


Fig. 7.3 Schematic overview of the function of microRNA. *DBR1* RNA debranching enzyme 1, *Ago* argonaute, *RISC* RNA-induced silencing complex

2014), and HDAC4 (Tuddenham et al. 2006). In traumatic-OA-induced models, intra-articular administration of miRNA140 inhibited OA progression, suggesting its potential as a therapeutic tool (Si et al. 2017).

7.4.2.2 miRNA17

miRNA17 is known for its decreased expression in OA cartilage (Zhang et al. 2022b). In vitro experiments suggested that miRNA17 negatively regulates MMP3, MMP13, ADAMTS5, and NOS2 expression. *miRNA17* knockout mice develop spontaneous OA and induction of miRNA17 agomir in traumatic-OA-induced models inhibits the progression of degeneration (Zhang et al. 2022b). Furthermore, HIF-1 α has also been suggested as a target of miRNA17 (Zhang et al. 2022b). However, as mentioned earlier, HIF-1 α maintains AC homeostasis (Okada et al. 2020), and the significance of the regulatory mechanism between miRNA17 and HIF-1 α requires detailed investigation.

Table 7.6 Summary of microRNA associated with articular cartilage homeostasis

Name	Effect for cartilage	Target genes	References
miRNA140	Protective	<i>ADAMTS5</i> , <i>DNPEP</i> , <i>RALA</i> , <i>HDAC4</i>	Wienholds et al. (2005), Tuddenham et al. (2006), Miyaki et al. (2009, 2010), Nakamura et al. (2011, 2012), Yamashita et al. (2012), Karlsen et al. (2014), Si et al. (2017)
miRNA17	Protective	<i>MMP3</i> , <i>MMP13</i> , <i>ADAMTS5</i> , <i>NOS2</i>	Okada et al. (2020), Zhang et al. (2022b)
miRNA101	Protective	<i>DNMT3b</i>	Kim et al. (2013), Zhang et al. (2017b)
miRNA379-5p	Protective	<i>YBX1</i>	Zhang et al. (2022a), Jee et al. (2018)
miRNA455-5p and -3p	Protective	<i>HIF-2α</i>	Ito et al. (2021), Swingler et al. (2012)
miR93-5p	Protective	<i>TCF4</i>	Xue et al. (2019), Ma et al. (2013)
miRNA126-5p	Offensive	<i>PGC1α</i>	Kim et al. (2021)
miRNA146a	Offensive	<i>CAMK2D</i> , <i>PPP3R2</i>	Yamasaki et al. (2009), Li et al. (2012), Hou et al. (2021), Zhou et al. (2022a), Zhang et al. (2017b), Guan et al. (2018)

7.4.2.3 miRNA101

miRNA101 has garnered research attention owing to its downregulated expression in OA cartilage (Kim et al. 2013). Intra-articular administration of miRNA101 can inhibit OA progression in traumatic-OA-induced mouse models. The mechanism involves targeting DNA methyltransferase 3 β (Dnmt3b) and preventing Dnmt3b from methylating and suppressing the expression of integrin- α 1 (Kim et al. 2013). Furthermore, miRNA101 silencing suppresses IL-1 β -stimulated downregulation of collagen type II and aggrecan expression in rat articular chondrocytes in vitro (Zhang et al. 2017b), indicating that further evaluation of the function of miRNA101 in articular chondrocyte homeostasis is needed.

7.4.2.4 miRNA379-5p

miR379-5p expression is induced by the PTHrP receptor agonist together with miR374-5p and miR503-5p in immature murine articular chondrocyte (IMAC) studies. miR379-5p has a cell proliferation-promoting effect and decreased expression of these miRNAs is involved in hypertrophy in endochondral ossification (Jee et al. 2018). Decreased expression of these miRNAs is also involved in promoting chondrogenic differentiation during endochondral ossification (Jee et al. 2018). miR379-5p expression is downregulated in OA AC compared with normal AC (Zhang et al. 2022a). It is predicted that miR379-5p targets and downregulates YBX1 in chondrocytes, reducing the activity of the PI3K/AKT signaling pathway, thus preventing cell death and maintaining the ECM-producing capacity (Zhang et al. 2022a). Intra-articular administration of miRNA379-5p in a traumatic OA-induced rat model inhibited OA progression, whereas administration of an

inhibitor caused more progressive OA (Zhang et al. 2022a). Therefore, miR379-5p may be a miRNA with a function in cartilage homeostasis.

7.4.2.5 miRNA455-5p and -3p

miR455, like miR140, is known to be highly expressed in AC (Swingler et al. 2012). miRNA455 is located within an intron of COL27A1, which encodes cartilage collagen (Swingler et al. 2012). The expression of both chains of miR455, -5p, and -3p, are induced by SOX9 (Ito et al. 2021). In miR455 knockout mice, OA changes with age (Ito et al. 2021). The target of miR455 in cartilage is hypoxia-inducible factor-2 α (HIF-2 α), and miR455 knockout mice have been rescued by HIF-2 α knockdown (Ito et al. 2021). Furthermore, intra-articular administration of miRNA455 in a traumatic OA-induced mouse model inhibited OA progression, indicating its potential as a therapeutic agent (Ito et al. 2021).

7.4.2.6 miRNA93-5p

miR93-5p is downregulated in osteoarthritic cartilage (Xue et al. 2019). miR93-5p overexpression in chondrocytes enhances cell viability, and intra-articular administration of this miRNA in a traumatic OA-induced mouse model inhibits OA progression (Xue et al. 2019). This is considered to be due to the fact that miR-93-5p directly targets the 3'-UTR of transcription factor 4 (TCF4) mRNA and suppresses its expression (Xue et al. 2019). TCF4 is involved in promoting the apoptosis of articular chondrocytes via the NF- κ B pathway (Ma et al. 2013), and suppressing this mechanism is expected to result in an anti-OA effect.

7.4.2.7 miRNA126-5p

miR-126-5p binds directly to and inhibits the 3'-UTR of PGC1 α (Kim et al. 2021). PGC1 α knockdown in chondrocytes selectively activates the mitochondrial autophagy (mitophagy) pathway by parkin RBR E3 ubiquitin protein ligase (PRKN) via elevated BCL2 and adenovirus E1B 19-kDa-interacting protein 3 (BNIP3) (Kim et al. 2021). Furthermore, BNIP3 overexpression stimulates mitophagy, leading to chondrolysis and chondrocyte death by upregulating the expression of chondrolytic enzymes, suggesting that the miR-126-5p-PGC1- α -BNIP3 pathway negatively affects cartilage homeostasis. This is supported by the fact that the OA-suppressing effect was seen by intra-articular injection of an antagonist of miR-126-5p in a traumatic-OA-induced mouse model (Kim et al. 2021).

7.4.2.8 miRNA146a

miR-146a is a miRNA that is strongly expressed in early OA cartilage tissue and chondrocytes upon IL1- β stimulation (Yamasaki et al. 2009; Li et al. 2012). Induction of miR-146a results in decreased levels of NRF2, increased expression of matrix-degrading enzymes, and decreased expression of the cartilage matrix in chondrocytes (Hou et al. 2021; Zhou et al. 2022a). It has also been confirmed that the onset of OA in miR146a knockout mice is slower than that in wild-type mice, which was observed both in the natural course and in a traumatic OA-induced model.

Moreover, the administration of an antagonist of miR146a in a traumatic OA-induced model of wild-type mice slowed OA onset (Zhang et al. 2017b). As miR-146a targets, calcium/calmodulin-dependent protein kinase II delta (Camk2d) and protein phosphatase 3, regulatory subunit B, beta isoform (Ppp3r2, also known as calcineurin B, type II) were identified (Zhang et al. 2017b). These targets are involved in the maintenance of Col2a1 and Sox9 expression in chondrocytes (Zhang et al. 2017b). Thus, miR-146a is a negative regulator of cartilage homeostasis, which promotes cartilage degeneration. However, in another report, analysis using miR146a knockout mice and cartilage-specific miR146a overexpressing mice showed opposite results, with progression of OA in the knockout mice and suppression of OA in the overexpressing mice compared to wild-type mice (Guan et al. 2018), indicating the importance of a more careful analysis of miR-146a function.

7.4.3 Circular RNAs (circRNAs)

CircRNAs are synthesized by back splicing, in which splicing occurs in the reverse direction during linear pre-mRNA maturation (Chen and Yang 2015). If splicing is performed normally, introns are removed from the pre-mRNA to form mRNA. However, back splicing can cause one or more exons to form a ring, yielding circRNA. Human circRNAs generally contain two or three exons. Their length is generally a few hundred bases, although some have been identified to have approximately 4000 bases. In addition, multiple circRNAs are produced from a single gene so that multiple transcripts can be produced by selective splicing of a single gene. At most, a dozen circRNAs have been identified from a single gene, but in most cases, only one or two are produced. Genome-wide analysis has predicted that circRNA expression accounts for 2–4% of mRNA expression (Szabo and Salzman 2016). circRNA has many potential functions, such as acting as a “sponge” for miRNAs and binding and sequestering other RBPs (Szabo and Salzman 2016) (Fig. 7.4). The most studied functional role of circRNAs in the AC is serving as miRNA sponges to suppress their downstream functions. A number of circRNAs have been reported (Liu et al. 2016, 2021, 2022; Wu et al. 2009, 2017a, b, 2020, 2021; Zhou et al. 2018a, b, 2019a, 2021a, b, 2022b; Zhang et al. 2020a, b, 2021c, d, e, 2022c, d; Ni et al. 2020; Chen et al. 2016, 2020b, c; Chang et al. 2019b, 2020; Jiang et al. 2021; Wang et al. 2015, 2020b, 2022; Apizi et al. 2021; Xi et al. 2021; Liu and Zhang 2021; Xu and Ma 2021; Shi et al. 2022; Jia and Wei 2021; Zheng et al. 2021; Chen and Xu 2021; Fu et al. 2021, 2022; Yang et al. 2015, 2021; Yi et al. 2016; Hui et al. 2016; Zhu et al. 2021b; Huang et al. 2021, 2022; Li et al. 2018, 2022a; Yu et al. 2022; Tang et al. 2022; Man et al. 2022; Ito et al. 2014; Luobu et al. 2022; Tonks 2006; Wahafu et al. 2022; He et al. 2022b; Shen et al. 2019, 2020; Tao et al. 2021; Ma et al. 2018, 2020; Ding et al. 2021; He and Cheng 2018; Wen et al. 2020; Gao et al. 2022), which can be broadly classified into those that promote OA and those that prevent OA. Table 7.7 summarizes these circRNAs, their target miRNAs, and the genes targetted by the miRNAs. In some of these reports, circRNAs were intra-articularly administered to animal models of traumatic OA to verify their therapeutic

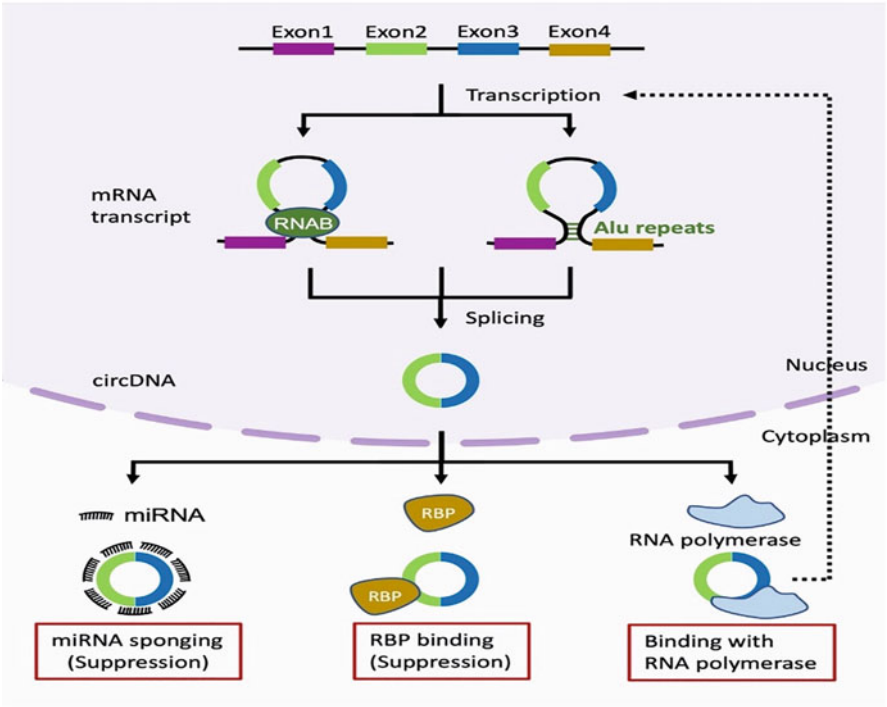


Fig. 7.4 Schematic overview of the function of circularRNA. *RBP* RNA-binding protein

effects (Zhou et al. 2019a, 2021a, 2022b; Wang et al. 2020b; Xu and Ma 2021; Yang et al. 2021; Hui et al. 2016; Huang et al. 2021, 2022; Li et al. 2022a; Tang et al. 2022; Shen et al. 2019, 2020; Tao et al. 2021). Further development in this field is expected.

7.4.4 Ubiquitination

Ubiquitin protein (Ub) is involved in post-translational modifications; Ub consists of three types of enzymes: activating enzyme E1, binding enzyme E2, and ligand enzyme E3. Ub is first activated by E1 and then transferred to the E2 binding enzyme. The E3 Ub ligase then simultaneously interacts with Ub-loaded E2 and the substrate protein to mediate isopeptide bond formation between the C terminus of Ub and substrate lysine 1 (Hershko and Ciechanover 1998). Ubiquitinated proteins undergo degradation, thereby losing their biological function (Fig. 7.5). Over 600 Ub E3 ligases and more than 100 deubiquitinating enzymes (DUBs) capable of removing Ub from substrates have been identified in the human genome (Grabbe et al. 2011). Here, we review ubiquitin enzymes that function in AC and are involved in homeostasis (Table 7.8).

Table 7.7 Summary of circular RNA associated with articular cartilage homeostasis

Name	Effect for cartilage	Target miRNA	Target of targetted miRNA	References
circRNA-CER	Offensive	miR-136	<i>MMP13</i>	Liu et al. (2016)
circRNA_0005105	Offensive	miR-26a	<i>NAMPT</i>	Wu et al. (2017a)
circRNA_Atp9b	Offensive	miR-138-5p	Inflammatory related gene	Zhou et al. (2018b)
circRNA_33186	Offensive	miR-127-5p	<i>MMP13</i>	Zhou et al. (2019a)
circRNA_CDH13	Offensive	miR-296-3p	<i>PTEN</i>	Zhou et al. (2021a)
circRNA_CDR1as	Offensive	miR-641	<i>FGF2</i>	Zhang et al. (2020a)
circRNA_PSM3	Offensive	miR-296-5p	Unknown	Ni et al. (2020)
circRNA_UBE2G1	Offensive	miR-373	<i>HIF1-α</i>	Chen et al. (2020b)
circRNA_HIPK3	Offensive	miR-124	<i>SOX8</i>	Wu et al. (2020)
circRNA_DHRS3	Offensive	miR-183-5p	<i>GREM1</i>	Jiang et al. (2021)
circRNA_RNF121	Offensive	miR-665	<i>MyD88</i>	Wang et al. (2020b)
circRNA_0001598	Offensive	miR-127-3p	<i>Unknown</i>	Apizi et al. (2021)
circRNA_0128846	Offensive	miR-127-5p	<i>NAMPT</i>	Liu et al. (2021)
circRNA_IQGAP1	Offensive	miR-671-5p	<i>TCF4</i>	Xi et al. (2021)
circRNA_0134111	Offensive	miR-515-5p and miR-224-5p	<i>SOCS1</i> , <i>CCL1/2</i>	Wu et al. (2021), Zhang et al. (2021c), Liu and Zhang (2021)
circRNA_0032131	Offensive	miR-502-5p	<i>TRX1</i>	Xu and Ma (2021)
circRNA_SEC24A	Offensive	miR-26b-5p and 142-5p	<i>DNMT3A</i> , <i>SOX5</i>	Zhang et al. (2021d), Shi et al. (2022)
circRNA_MSR	Offensive	miR-643	<i>MAP2K6</i>	Jia and Wei (2021)
circRNA_0116061	Offensive	miR-200-3p	<i>SMURF2</i>	Zheng et al. (2021)
circRNA_0008956	Offensive	miR-149-5p	<i>NAMPT</i>	Fu et al. (2021)
circRNA_SPG11	Offensive	miR-665	<i>GREM1</i>	Yi et al. (2016)
circRNA_RSU1	Offensive	miR-93-5p	<i>MAP3K8</i>	Yang et al. (2021)
circRNA_0136474	Offensive	miR-766-3p	<i>DNMT3A</i>	Zhu et al. (2021b), Wu et al. (2017b)
circRNA_0092516	Offensive	miR-337-3p	<i>PTEN</i>	Huang et al. (2021, 2022)
circRNA_0000423	Offensive	miR-27-3p	<i>MMP13</i>	Li et al. (2022a)
circRNA_0020014	Offensive	miR-613	<i>ADAMTS5</i>	Yu et al. (2022)

(continued)

Table 7.7 (continued)

Name	Effect for cartilage	Target miRNA	Target of targetted miRNA	References
circRNA_NFKB1	Offensive	This cirRNA works with ENO1 and affect the NF-kb pathway		Tang et al. (2022)
circRNA_RHOT1	Offensive	miR-142-5p	<i>CCND1</i>	Man et al. (2022)
circRNA_SCAPER	Offensive	miR-140-3p	<i>EZH2</i>	Luobu et al. (2022), Chen et al. (2016)
circRNA_0128846	Offensive	miR-940	<i>PTPN12</i>	Fu et al. (2022)
circRNA-PRKCH	Offensive	miR-502-5p	<i>ADAMTS5</i>	Liu et al. (2022)
circRNA_0005526	Offensive	miR-142-5p	<i>TCF4</i>	Wahafu et al. (2022)
circRNA_0043947	Offensive	miR-671-5p	<i>RTN3</i>	He et al. (2022b)
circRNA_SERPINE2	Protective	miR-1271-5p	<i>ERG</i>	Shen et al. (2019)
circRNA_ANKRD36	Protective	miR-599	<i>CASZ1</i>	Zhou et al. (2021b)
circRNA_9119	Protective	miR-26a	<i>PTEN</i>	Chen et al. (2020c)
circRNA_CDK14	Protective	miR-125a-5p	<i>SMAD2</i>	Shen et al. (2020)
circRNA_3503	Protective	miR-181c-3p and hsa-let-7b-3p	<i>SOX9</i>	Tao et al. (2021)
circRNA_0001103	Protective	miR-375	<i>SIRT1</i>	Zhang et al. (2021e)
circRNA_0045714	Protective	miR-331-3p	<i>PIK3R3</i>	Ding et al. (2021)
circRNA_Ph3	Protective	miR-93-3p	<i>FOXO</i>	Wang et al. (2022)
circRNA_PDE4D	Protective	miR-4306	<i>SOX9</i>	Gao et al. (2022)
circRNA_SPI1_005	Protective	miR-370-3p	<i>MAP3K9</i>	Zhou et al. (2022b)
circRNA_LRP1B	Protective	miR-34a-5p	<i>NRF1</i>	Chang et al. (2020)
circRNA_0110251	Protective	miR-3189-3p	<i>SPRY1</i>	Zhang et al. (2022d)

7.4.4.1 AXIN

AXIN functions in ubiquitination of β -catenin in the Wnt/ β -catenin pathway. Cartilage-specific AXIN knockout mice exhibit an OA phenotype due to activation of the Wnt/ β -catenin and FGF/ERK1/2 signaling pathways in AC (Zhou et al. 2019b). Ubiquitination and destabilization of AXIN are enhanced in OA chondrocytes. AXIN is a ubiquitinating enzyme that functions as a positive regulator of AC homeostasis (Ba et al. 2020).

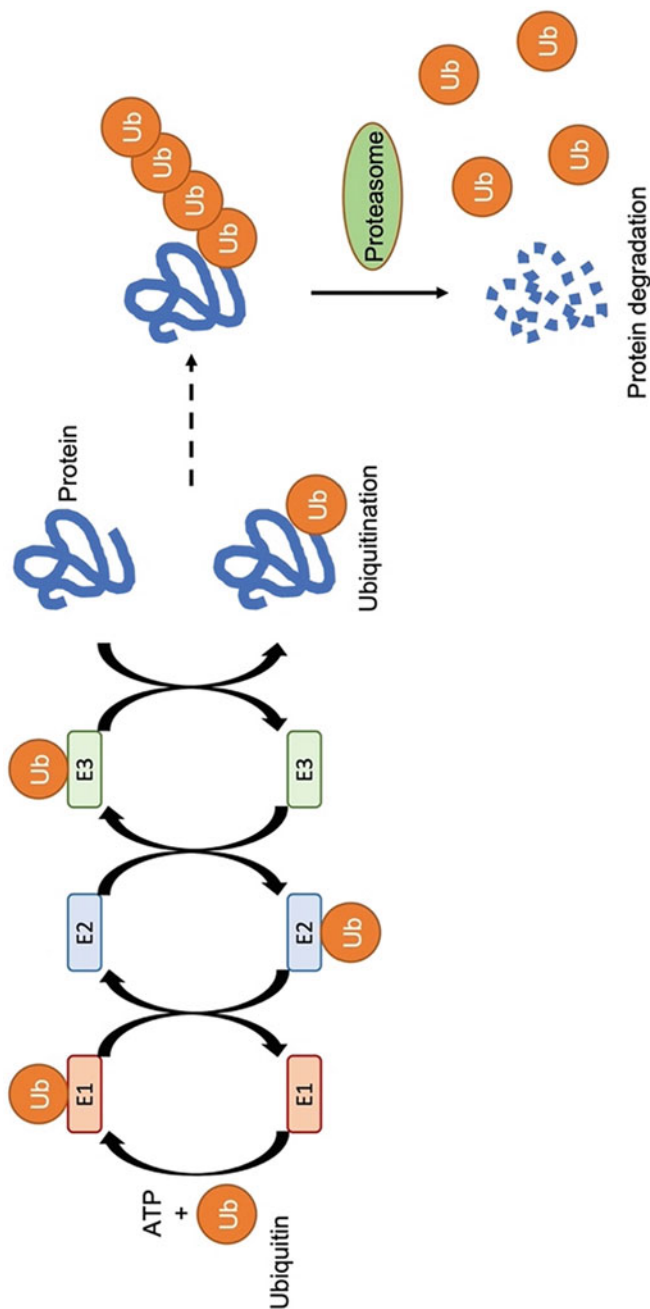


Fig. 7.5 Schematic overview of the function of ubiquitinases. *E1* ubiquitin activation enzyme1, *E2* ubiquitin activation enzyme2, *E3* ubiquitin activation enzyme3

Table 7.8 Summary of ubiquitinases associated with articular cartilage homeostasis

AXIN	Protective	Ubiquitination of β -catenin	Zhou et al. (2019b), Ba et al. (2020)
UBE2M	Offensive	Ubiquitination of AXIN	Ba et al. (2020)
PARKIN	Protective	Ubiquitination of MFN2	Qiu et al. (2017), Ansari et al. (2018), Xu et al. (2020)
FBXO6	Protective	Ubiquitination of MMP14	Wang et al. (2020c), Merry et al. (2010)
AURKA	Offensive	Ubiquitination of SOD2	Yang et al. (2019)
WWP2	Protective	Ubiquitination of ADAMTS5	Bernassola et al. (2008), Rotin and Kumar (2009), Mokuda et al. (2019)

7.4.4.2 Ubiquitin-Conjugating Enzyme E2 M (UBE2M)

UBE2M is a ubiquitin enzyme that is highly expressed in human OA cartilage tissue (Ba et al. 2020). The target of the UBE2M is AXIN. Therefore, UBE2M is expected to promote OA by activating OA chondrocyte apoptosis and the Wnt/ β -catenin pathway through AXIN downregulation, suggesting that UBE2M is a negative regulator of AC homeostasis (Ba et al. 2020).

7.4.4.3 PARKIN

PARKIN is an E3 ubiquitin ligase involved in Parkinson's disease (Qiu et al. 2017). In chondrocytes, parkin ubiquitin ligase activity is required for PARKIN-mediated depolarization/removal of damaged mitochondria, and it reduces ROS levels and inhibits apoptosis (Ansari et al. 2018). In vitro experiments using chondrocytes showed that PARKIN inhibited OA progression by targeting Mitofusion 2 (MFN2) and suppressing inflammation (Xu et al. 2020).

7.4.4.4 F-Box Protein 6 (FBXO6)

FBXO6 is a ubiquitin E3 ligase that binds to high-mannose N-linked glycoproteins and ubiquitinates them (Wang et al. 2020c; Merry et al. 2010). FBXO6 expression is decreased in human OA cartilage and the cartilage of a traumatic OA-induced mouse model (Wang et al. 2020c), and global *Fbxo6* knockout mice and cartilage-specific *Fbxo6* knockout mice with the *Col2-creERT2* strain exhibit knee OA progression with aging (Wang et al. 2020c). FBXO6 inhibits MMP14-dependent proteolytic activation of MMP13 by ubiquitinating MMP14. Through this mechanism, induction of *Fbxo6* expression in AC in a traumatic OA-induced mouse model showed a suppressive effect on OA (Wang et al. 2020c). Therefore, FBXO6 may be a positive regulator of AC homeostasis.

7.4.4.5 Aurora Kinase A (AURKA)

AURKA is a ubiquitinase that is highly expressed in OA cartilage tissues and chondrocytes (Yang et al. 2019). Stimulation with IL1-B with AURKA suppression in chondrocytes suppressed the upregulation of *Mmp13* and *Asmts5* and

downregulation of *Col2a1* and *Acan* (Yang et al. 2019). Administering ShAURKA to traumatic OA-induced rat models suppressed OA progression because AURKA targets superoxide dismutase 2 (SOD2) for ubiquitination and mitochondrial dysfunction due to decreased SOD2 expression by AURKA, which is involved in OA pathogenesis (Yang et al. 2019). Thus, AURKA is considered a negative regulator of AC homeostasis.

7.4.4.6 WW Domain-Containing Protein 2 (WWP2)

Wwp2 is a member of the C2-WW-HECT family (NEDD4 family) of E3 ubiquitin ligases (E3), which receives ubiquitin from the E2 enzyme and transfers it to specific lysine residues on the substrate (Bernassola et al. 2008; Rotin and Kumar 2009). It is also known as the host gene for miRNA140 as described above. WWP2 regulates ADAMTS5 expression by regulating RUNX2 expression (Mokuda et al. 2019). Although development appeared to occur normally in *Wwp2* knockout mice, RUNX2 and ADAMTS5 expression was enhanced in 6-month-old articular chondrocytes, indicating progressive OA. Furthermore, WWP2 had an inhibitory effect on OA progression when administered to a traumatic OA mouse model, suggesting that WWP2 is a positive regulator of AC homeostasis (Mokuda et al. 2019).

7.5 Reactive Oxygen Species

Cartilage is a tissue with poor blood flow, and mitochondria play an important role in chondrocyte survival under hypoxic conditions (Terkeltaub et al. 2002; Blanco et al. 2011). Considering that mitochondrial dysfunction is associated with chondrocyte apoptosis (Kim and Blanco 2007), the maintenance of mitochondrial function is major therapeutic targets for OA. Proteomic analysis of mitochondrial proteins in normal and OA cartilage in human articular chondrocytes showed cluster changes characterized by alterations in energy production, maintenance of mitochondrial membrane integrity, and detoxification of free radicals (Ruiz-Romero et al. 2009). We review the factors associated with the maintenance of mitochondrial function (Table 7.9).

7.5.1 Superoxide Dismutase 2 (SOD2)

SOD2 is an enzyme that protects mitochondria from oxidative stress (Turrens 2003). SOD2 is downregulated in OA cartilage, suggesting that this factor may be involved in OA pathogenesis (Ruiz-Romero et al. 2009; Aigner et al. 2006; Scott et al. 2010; Koike et al. 2018). SOD2 expression is downregulated in traumatic OA-induced mouse models, thus promoting mitochondrial superoxide formation, whereas SOD2 deficiency increases mitochondrial superoxide in chondrocytes, leading to cartilage degeneration (Koike et al. 2015). Thus, maintenance of SOD2 expression is expected to be important for the maintenance of AC homeostasis.

Table 7.9 Summary of factors associated with the maintenance of mitochondrial function

Name	Effect for cartilage	Function	References
SOD2	Protective	Protection of Mitochondria from oxidative stress	Ruiz-Romero et al. (2009), Turrens (2003), Aigner et al. (2006), Scott et al. (2010), Koike et al. (2015, 2018)
NRF2	Protective	Activation of ERK1/2 pathway, transcriptional regulation of SOX9	Kensler et al. (2007), Pajares et al. (2017), Wruck et al. (2011), Cai et al. (2015), Khan et al. (2018), Kubo et al. (2022), Song et al. (2021)
PRDX	Protective	Maintenance of the expression of Peroxiredoxin	Ding et al. (2017), Collins et al. (2021), Li et al. (2022b)
MFN2	Offensive	Metabolic shift to mitochondrial respiration	Xu et al. (2020)

7.5.2 Nuclear Factor (Erythroid-Derived 2)-Like 2 (NRF2)

NRF2 is an important intracellular redox regulator responsible for the regulation of protective factors involved in the recognition and removal of damaged proteins and organelles (Kensler et al. 2007; Pajares et al. 2017). Drug- or surgery-induced OA in *Nrf2* knockout mice resulted in more severe cartilage destruction than in wild-type mice (Wruck et al. 2011; Cai et al. 2015). An in vitro study showed that NRF2 activates ERK1/2 and its downstream targets, ELK1, P70S6K, and P90RSK and significantly suppresses IL-1 β -induced ROS generation and activation of external and internal apoptotic pathways (Khan et al. 2018). Moreover, NRF2 binds to an antioxidant response element (ARE) in the promoter region of *SOX9* and increases its transcriptional activity, suggesting that regulating *SOX9* expression is another function of NRF2 (Kubo et al. 2022). Intra-articular injection of *Nrf2* overexpressing lentivirus into a traumatic OA-induced mouse model reduced MMP13 expression in cartilage, serum TNF- α , IL-1 β , and IL-6 levels, and inhibited OA progression (Song et al. 2021).

7.5.3 Peroxiredoxins (PRDX)

PRDX1 is an antioxidant protein, among which PRDX family is mainly found in the cytoplasm, is involved in interactions with several ROS-dependent effectors, and plays an important role in cell survival (Ding et al. 2017). Sirtuin 6 (SIRT6) is a nuclear-localized protein deacetylase known to maintain redox homeostasis in chondrocytes by maintaining peroxiredoxin expression; however, its activity is reduced in OA chondrocytes, resulting in reduced *Prdx* expression (Collins et al. 2021). An in vivo experiment showed that intra-articular administration of the antioxidant methylene blue maintained *Nrf2* and *Prdx1* expression in AC and inhibited OA progression (Li et al. 2022b).

7.5.4 Mitofusion 2 (MFN2)

MFN2 is an important regulator of mitochondrial fusion, cellular metabolism, autophagy, and apoptosis. In chondrocytes, MFN2 expression gradually increases with age, resulting in a metabolic shift toward mitochondrial respiration; however, MFN2 knockdown in chondrocytes reverses age-related metabolic changes (Xu et al. 2020). As described above, the target gene of PARKIN, an E3 ubiquitin ligase, is MFN2, which may be a candidate therapeutic target for OA (Xu et al. 2020).

7.6 Conclusion

This chapter aims to support improved understanding of the mechanisms of AC development and homeostasis for application to AC regenerative medicine. Various factors reported in the field of molecular biology research from the 1990s to the present are comprehensively reviewed herein. For clinical application, it will be important to combine these factors with the development of novel tools to yield highly efficient and tissue-specific induction and delivery. We hope that AC regeneration therapy will be supported through the consolidation of these findings.

Acknowledgments We thank all the Asahara Lab members for their helpful discussions.

Funding AMED-CREST from AMED (Japan Agency for Medical Research and Development) (JP21gm0810008 to H.A.)

JSPS KAKENHI (grant numbers: 20H05696 and 21K19403 to H.A.)

National Institutes of Health (Grant numbers: AR050631 and AR065379 to H.A.)

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Extracellular Matrix Biomimicry for Cartilage Tissue Formation

8

Raminta Vaiciuleviciute, Jolita Pachaleva, Ursule Kalvaityte, Viktorija Aleksuk, Ilona Uzieliene, Ali Mobasher, and Eiva Bernotiene

Abstract

Development of biomimetic constructs for cartilage tissue regeneration has become an increasing field of interest in today's biomedical research. Both, synthetic and natural compound-based scaffolds are being used for biomimetic construct formation, and their mix, with polylactic acid (PLA), poly(ethylene glycol) (PEG), etc., being on the lead of most commonly used synthetic materials and cartilage extracellular matrix (ECM) proteins, such as collagens, hyaluronic acid (HA), glycosaminoglycans (GAGs), and proteoglycans, are the most popular ones for developing natural scaffolds/hydrogels for cartilage tissue engineering purposes. Most important properties for both types of scaffolds include fluid absorption, piezoelectric properties, surface parameters, porosity, electrical conductance, stiffness, and wettability. Synthetic materials are mostly favorable for their stiffness and mechanical stability, while natural molecules are advantageous for mimicking the in vivo environment, stimulating cell differentiation processes

R. Vaiciuleviciute · J. Pachaleva · U. Kalvaityte · V. Aleksuk · I. Uzieliene · E. Bernotiene (✉)
Department of Regenerative Medicine, State Research Institute Centre for Innovative Medicine,
Vilnius, Lithuania
e-mail: eiva.bernotiene@imcentras.lt

A. Mobasher
Department of Regenerative Medicine, State Research Institute Centre for Innovative Medicine,
Vilnius, Lithuania

Research Unit of Health Sciences and Technology, Faculty of Medicine, University of Oulu, Oulu,
Finland

World Health Organization Collaborating Center for Public Health Aspects of Musculoskeletal
Health and Aging, Université de Liège, Liège, Belgium

Department of Joint Surgery, First Affiliated Hospital of Sun Yat-sen University, Guangzhou,
Guangdong Province, China

and possessing biodegradability. In order to achieve the highest biomimicry of a construct to be used for regenerative purposes of cartilage tissue, optimal conditions of scaffold synthesis and its application techniques should be optimized. To date, no effective scaffold system exists, which would overcome every difficulty, and challenges of developing a qualitative construct remain high. However, a number of studies demonstrated promising results of using different kinds of scaffolds, suggesting their potential application for clinical trials. This chapter deals with synthetic, natural, and their mixed scaffolds, as well as their properties and functions for stimulating cellular responses for cartilage regeneration purposes. We observe scaffolds' basic chemical/biological properties, their effects on chondrogenic differentiation, and physical stimuli, such as mechanical and electrical stimulations. We assume this chapter will bring novel insights for early and advanced scaffold developers and will help indicate the most important properties of a scaffold system required for cartilage tissue regeneration.

Keywords

Cartilage · Biomimetic scaffolds · Synthetic scaffolds · Natural scaffolds · Mechanical/electrical stimulation

8.1 Introduction

Cartilage is aneural, avascular, and alymphatic tissue, nutrition relying on diffusion from surrounding tissues, which are the reasons for its limited spontaneous regeneration abilities (Armiento et al. 2019). The degradation and loss of articular cartilage and the lack of regenerative properties in mature cartilage tissue highlight an unmet medical need and an opportunity for the development of new innovations and regenerative treatment strategies. Osteoarthritis (OA) is the most common form of arthritis globally, affecting more than 500 million people (Liem et al. 2020). OA development is driven by catabolic and proinflammatory agents that result in overproduction of proteolytic enzymes, leading to cartilage degeneration (Wei et al. 2021). Currently, treatments can be divided into two major types: non-surgical, which includes non-pharmacological and pharmacological strategies, and surgical intervention. However, most of the currently available pharmacological treatments provide only symptom management at most and cause various gastrointestinal, renal, and cardiovascular side effects. Many years or even decades may pass until the joint is rendered totally dysfunctional. At this point, total joint arthroplasty is the only currently available option but this strongly invasive procedure is applied only at the end stages of OA and requires long recovery time. Therefore, tissue engineering (TE) techniques, using biomimetic scaffolds, offer viable solutions for chondral and osteochondral defects, may prevent progression of joint destruction, and restore articular cartilage structure and function (Kwon et al. 2019; Makris et al. 2015).

The term biomimicry describes innovations inspired by nature (Benyus 1997). Biomimicry in biomedical research specifically aims to recapitulate naturally occurring processes, forms, and functions (Qu et al. 2015). For cartilage engineering, recapitulation of a complex multi-layered tissue, mimicking different structural, mechanical, and functional properties, is crucial. There are three main strategies to tackle the goal of mimicking the cartilage, seeking to restore damaged tissue. First is to induce regeneration by implanting engineered matrices and allowing immigration of cells from surrounding tissues. The second is to inject autologous, allogenic, or xenogeneic cells directly into the joint cavity, with the advantage of having the ability to manipulate the cells prior to injection. However, in order to sufficiently mimic cartilage tissue, a proper layering and orientation of ECM and cells are necessary, while the injection of cells alone cannot provide any structure. The third strategy is a combination of both, where the cells are seeded onto the scaffold and the whole construct implanted. This enables cells to synthesize new ECM, and by the time the scaffold degrades, the new tissue is formed (Fu et al. 2020; Kuo and Tuan 2003). A complex selection of materials and physicochemical parameters of a scaffold plays an important part in the cartilage TE process. There is a wide variety of materials to choose, including natural, synthetic, or a combination of several substances. Natural materials can be either ECM-based (collagen, chondroitin sulfate (CS), hyaluronic acid (HA), other glycosaminoglycans (GAGs), decellularized cartilage) or non-ECM-based biomimetic (silk, alginate, chitosan, gelatin, hydroxyapatite (HAP)) constructs. The synthetic scaffold materials can be divided into hydrophilic materials (bioactive glass, etc.) and hydrophobic materials (poly (glycolic acid) (PGA), poly(lactic acid) (PLA), poly(ϵ -caprolactone) (PCL), etc.). Physicochemical and biological properties of ECM play a crucial role in the cartilage regeneration process and its mimicry becomes an important criterion for the choice of materials. These parameters include fluid absorption, piezoelectric properties, surface parameters, porosity, electrical conductance, stiffness, and wettability.

This chapter focuses on different cartilage TE approaches, the materials used, and the properties that should be taken into consideration in order to achieve the highest biomimicry of native cartilage tissue.

8.2 Physicochemical and Biological Properties of Biomimetic Constructs

The formulation of scaffold must recapitulate natural cartilage structure and mechanical properties. Biomimicry of various physicochemical characteristics has to be considered in order to find optimal parameters for cellular response. The main properties, essential for cell biocompatibility, are listed in Fig. 8.1. These properties mostly depend on scaffold composition and can be modified by changing manufacturing techniques, using different polymers.

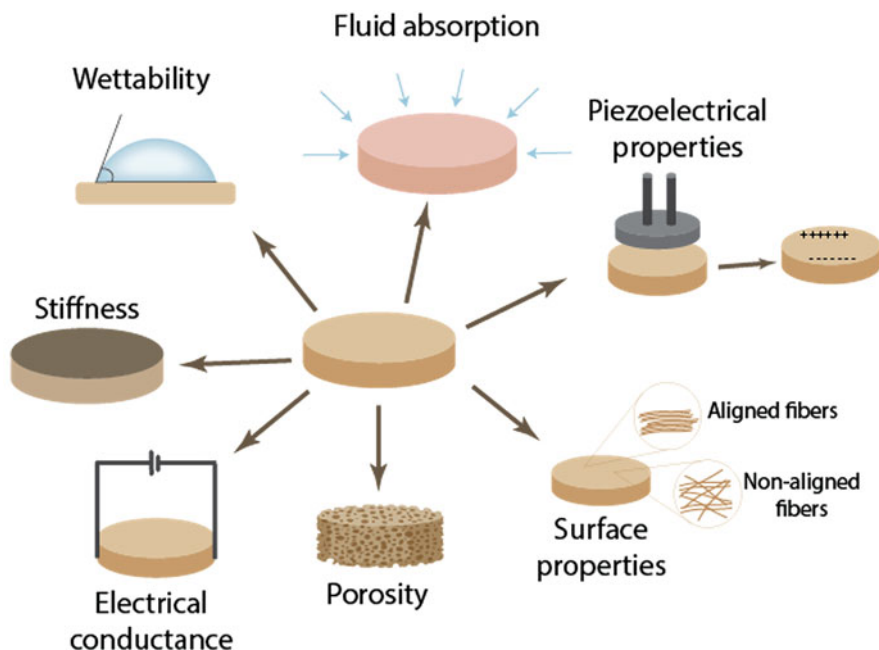


Fig. 8.1 Physicochemical properties of biomimetic scaffolds

8.2.1 Physicochemical Properties

8.2.1.1 Stiffness

Cartilage is a non-uniform load-bearing tissue—superficial zone resists shear stresses while middle zone provides a tensile network which prevents ECM expansion during compression (Lakin et al. 2017). Stiffness of cartilage varies between 0.4 and 2 MPa (Setton et al. 1999), while it differs depending on layer (e.g., pericellular matrix is 43–240 kPa) and condition (on the first stage of OA—1.14 to 1.3 MPa, second stage—1.02 to 1.2 MPa, and third stage—0.82 to 1.2 MPa) (Alexopoulos et al. 2005; Ihnatouski et al. 2020). Chondrocytes and mesenchymal stromal cells (MSCs) can detect substrate stiffness via mechanoreceptors (e.g., integrin $\beta 1$) and direct differentiation into chondrogenic or osteogenic way (Oliveras-Navarrete et al. 2017). Studies have shown that scaffolds with even lower stiffness than cartilage might induce osteogenic differentiation. $\sim < 3\text{--}4$ kPa synthetic hydrogels facilitated cell condensation and chondrogenic differentiation, stiffer ones (> 10 kPa) increased cell proliferation, non-cartilaginous phenotype, and stiffness > 100 kPa enhance osteogenic differentiation, making it more difficult to prognose cellular response based on scaffold stiffness (Arora et al. 2016; Ren et al. 2016; Zhan 2020). Natural materials have lower stiffness than natural cartilage (e.g., chitosan is 1.57–1.66 kPa, scaffold made from 7.5% gelatin— 1.2 ± 0.4 kPa, chitosan-gelatin mixture—1.15–3.4 kPa) but they risk not to withstand mechanical loading and it limits their

application in TE (Qu et al. 2019). To solve this problem, stiffness can be modified by changing concentrations and mixing ratios of biomaterials or incorporating polymers. Compressive modulus of gelatin/hyaluronic acid scaffolds increased as the HA ratio increased (Afewerki et al. 2019; Büyüköz et al. 2018). Synthetic scaffolds have higher stiffness mostly depending on material origin and porosity of a construct. Small pore (0.2 mm) 3D bio-printed PCL and PCL-HAP scaffolds had significantly higher values of Young's modulus compared to medium pore (0.5 mm) and large pore (0.9 mm) (Bittner et al. 2019). Stiffness can also be modulated by cross-linking protocols and incorporation of polymers (Kim et al. 2017). Other parameters, such as pore strand size, strand spacing, and strand orientation, affect scaffold porosity and compressive strength as well (Olubamiji et al. 2016). Although there are different ways to modulate scaffold stiffness, but for the most part, mechanical properties that match those of natural cartilage are not achieved yet (Little et al. 2011).

8.2.1.2 Porosity

Cartilage ECM has high porosity with relatively low number of cells and a high amount of matrix, and the avascularity creates hypoxic conditions with levels of oxygen ranging from 9% to 2% in deepest zones (Sieber et al. 2020). Decellularized human cartilage scaffolds had porosity of 89.3% (Yang et al. 2008). Porous materials are usually used for cartilage biomimicry as they allow fast ingrowth of cartilage-forming elements. Scaffold porosity ensures cell–cell interactions and diffusion of nutrients, where higher pores provide a space for cell migration and proliferation while micropores are crucial for cell attachment (Ren et al. 2016; Sultan and Mathew 2018). Gradient porosity and pore size scaffolds have already been created by electrospinning or 3D printing but creation of oxygen gradient in a scaffold remains challenging; hypoxic conditions are thus usually created externally (Bornes et al. 2015; Sultan and Mathew 2018; Timnak et al. 2018; Zubillaga et al. 2020). It has been demonstrated that chondrocytes prefer scaffolds with a pore size between 250 and 500 μm due to the sufficient space provided for oxygen and nutrients maintenance for cell growth and chondrogenesis (Lien et al. 2009). In recent studies, electrospinning was used as a promising technique for constructing nanofibrous scaffolds with interconnected porosity. Porous-architecture of these scaffolds is highly similar to the native extracellular matrix (ECM) (Chen et al. 2022; Sharifi et al. 2020). Scaffolds could be fabricated by using a freeze-drying process to achieve the required pore size and structure. Freezing produces a network of ice crystals surrounded by fibers. Sublimation of the ice crystals leads to the formation of highly porous scaffolds (Matsiko et al. 2015). Freeze-dried SF/Ch-GI-CS scaffolds successfully induced chondrogenic differentiation of hMSCs derived from umbilical cord blood (UCB) (Agrawal and Pramanik 2019). Porous, biomimetic scaffolds from collagen type I (Col I), and GAG supported chondrogenesis of adult rat MSCs in vitro (Farrell et al. 2006). A highly porous alginate foam scaffold with CS fabricated by the freeze-drying method has a high porosity ~93–95% with a mean pore size of $197 \pm 61 \mu\text{m}$. It maintained the chondrogenic phenotype of human articular chondrocytes and increased differentiation of BMMSC (Huang et al. 2015).

Nava et al. showed that density of primary bovine articular chondrocytes increased up to 50% with increased pore size (from 75 to 175 μm) and porosity (from 71 to 84%) of poly-L-lactide-*co*-trimethylene carbonate scaffolds in vitro (Nava et al. 2016). Moreover, another study showed that canine chondrocytes, seeded on PLA scaffolds with porosity similar to articular bovine cartilage, had highest viability and ECM production on macro/microporous scaffold with lower pore size (250 μm comparing to 400 μm) (El-Ayoubi and DeGrandpré 2011). Porosity of the scaffold is important for external stimuli, especially mechanotransduction. It is known that scaffolds with a lower porosity lack mechanical stability; meanwhile, cells that attach on the struts will receive lower strain (Hendrikson et al. 2017). Many studies demonstrated that porosity of scaffold mimicking average porosity of natural cartilage and variation of pore size is easily achievable while gradient porosity is still a challenge.

8.2.1.3 Surface Properties

The superficial zone of an articular cartilage has the tightly packed aligned collagen fibrils, providing high resistance to shear (Lin et al. 2017), while in the middle zone fibers are randomly aligned (Girão et al. 2018). Collagen fibrils of human articular cartilage have a diameter of about 33 nm and human OA cartilage has collagen fibrils of 88 nm diameter (Maniwa et al. 2019). Scaffolds do not fully mimic the surface of cartilage as they have to provide an optimal environment for cell attachment and ingrowth. Therefore, roughness of surface, fiber diameter, and alignment are the most important parameters for scaffold formulation. It was shown that aligned orientation of collagen-polyvinyl alcohol (PVA) nanofibers and ECM-derived scaffolds increased stiffness of construct (Jia et al. 2012; Lin et al. 2017). Hydrogel-like or fiber-like chitosan surface on PLA scaffold have different effects on chondrogenesis. A hydrogel-like coating showed not efficient cell condensation or matrix maturation, but improved chondrogenic phenotype of BMMSC, while a fiber-like coating induced cell–matrix interactions, reducing hypertrophy and resulting in a more stable hyaline-like cartilage (Magalhães et al. 2015). Mild surface roughness is favorable for cell adhesion and induction of chondrogenic phenotype, while surface of cartilage from OA patients has significantly higher roughness (Youssef et al. 2021). Roughness and hydrophilicity of PCL scaffold can be increased by enzymatic treatment without changing compressive strength and it results in induction of porcine chondrocytes chondrogenesis, higher expression of cartilage-specific gene (i.e., Col II), and the increased production of GAGs (Kosorn et al. 2019). Contrary to collagen fibers in human cartilage, PCL and PLA microfibers stimulated human MSC chondrogenic differentiation in vitro more effectively in comparison with nanofiber scaffolds (Bean and Tuan 2015; Shanmugasundaram et al. 2011). According to these studies, fibrous scaffolds with align orientation show best results while optimal fiber diameter is not clear.

8.2.1.4 Piezoelectric Properties

Analogously to native human cartilage, piezoelectric materials can transduce the mechanical pressure to the electrical signals, this process is called direct

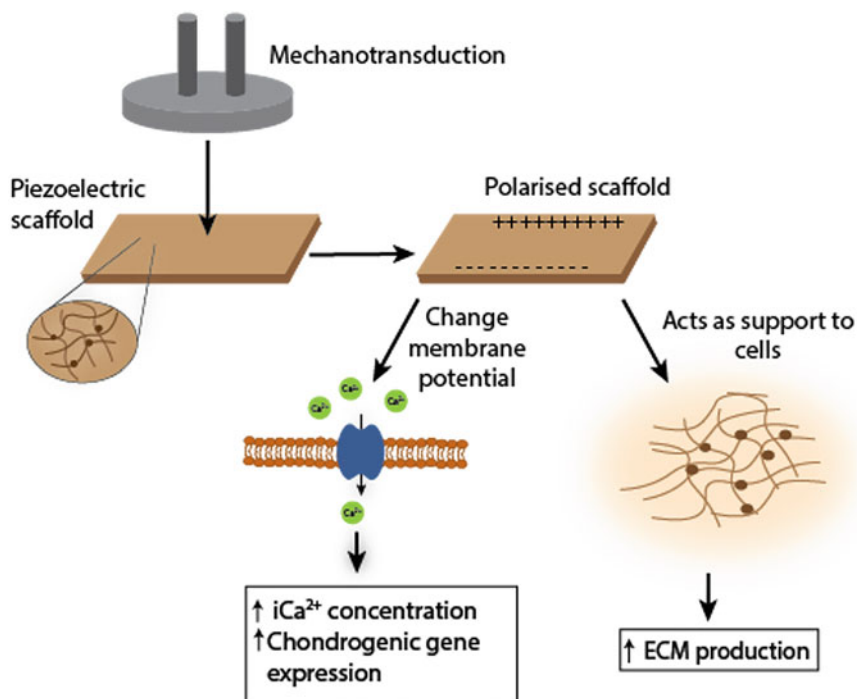


Fig. 8.2 The effect of piezoelectric scaffold on chondrogenesis

piezoelectric effect (Fig. 8.2). Induced electric signal, which is similar to external electrical signals, changes membrane potential and opens voltage-gated calcium channels (VGCC) in cell membrane, causing iCa^{2+} influx, resulting in alterations of gene transcription (Jacob et al. 2018; Przekora 2019). This system does not need an external power source and can induce cell differentiation without adding any growth factors. The reverse process is transducing electrical signals to mechanical deformation, that is called converse piezoelectric effect (Lai et al. 2021). Moreover, piezoelectric scaffolds also act in a passive way supporting cells and enhancing production of ECM (Ribeiro et al. 2015). Col I fibrils are capable of generating an electric potential up to tens of millivolts, making bones and tendons piezoelectric with a polarization along the fibril length (Minary-Jolandan and Yu 2009). Col II, which is a main constituent of human articular cartilage, also is piezoelectric with one-third lower piezoelectric coefficient than Col I.

Electrical output depends not only on scaffold material, but also on surface morphology. Recent data showed that PLA wrinkled fibers exhibit better piezoelectric characteristics and a larger electrical output than the porous fibers. The orientation of fibers also affects electrical output—output voltage along the 90° orientation was higher than that in the 0° orientation. Voltage output of piezoelectric scaffold can differ based on fabrication method—electric fields of annealed poly(vinylidene fluoride)-trifluoroethylene (PVDF-TrFE) scaffold was 1 V/mm while electrospun

was 20 mV/mm (Damaraju et al. 2017). Electric output can be increased by adding low concentration (0.35 mg/mL) of reduced graphene oxide (rGO) which enhances electric output in PLA scaffolds and it has larger elasticity in comparison with higher concentration of rGO (Lai et al. 2021). As piezoelectric scaffolds mimic mechanical load transduction into electric signals in cartilage, the application of these scaffolds for cartilage engineering is not clear yet, while more examples will be discussed in Sect. 8.3.

8.2.1.5 Electrical Conductivity

Many cellular processes rely on endogenous electrical currents in human organisms making electroconductive scaffolds beneficial for tissue formation and maintaining microenvironment (Marsudi et al. 2021). Electroconductive scaffolds can be generated from any biopolymer by introducing ionic functional groups such as carboxylic, sulfonic, amine, etc., or made of conductive polymers such as polyacetylene, poly(*para*-phenylenevinylene), HSiline (PANI), polythiophenes, and polypyrrole (PPy). Conductive scaffolds can also be fabricated by using carbon nanotubes, graphene, conductive nanofibers, metallic nanoparticles (Nekounam et al. 2021). Electroconductive scaffolds are widely used in chondrogenesis under electrical stimulation (ES) studies, which will be discussed with more details in Sect. 8.3.

8.2.1.6 Fluid Absorption

As cartilage is an avascular tissue, nutrients are transported to chondrocytes by diffusion of synovial fluid in and out of cartilage, absorption of large molecules (serum albumin) can be facilitated by mechanical compression (O'Hara et al. 1990). Fluid or water absorption by biomimetic scaffolds is crucial for providing medium and nutrients to cells in the scaffold. Absorption mostly depends on scaffold microarchitecture—pore size and maintenance of 3D structure (Liang et al. 2010). Scaffold absorbed water can be classified as total absorbed water (measured by weighting dry and wet scaffolds) and substrate-bound water (e.g., collagen has abundant water-binding amino acids) (Irawan et al. 2018). Natural biomaterials, such as collagen and gelatin, have higher fluid absorption than synthetic ones (Utomo et al. 2019). Gelatin scaffold ability to absorb fluid was used as a way to physically immobilize soluble growth factors, such as hyaluronan, transforming growth factor β 3 (TGF- β 3). Results showed that absorbed growth factors were consumed during 21 day of chondrogenesis study and it increased collagen content in scaffold without affecting sGAG content and showed highest expression of SOX9, aggrecan, COL2A1 mRNA expression in primary human chondrocytes (Klangjorhor et al. 2014). Higher fluid absorption is favorable in biomimetic scaffolds and natural materials are used to achieve better results.

8.2.1.7 Surface Wettability

Biomimetic scaffolds should be hydrophilic to contribute cell–surface interactions and maintain flattened phenotype (Kim et al. 2007). Scaffold surface wettability is a parameter describing scaffold hydrophobicity (contact angle $>90^\circ$)/hydrophilicity

(contact angle $<90^\circ$). It is known that hydrophobicity of scaffolds negatively affects cell adhesion and proliferation which limits their application possibilities, so, for TE polymers ought to have contact angle below 100° (Lipovka et al. 2020). Natural polymers usually have higher surface wettability than synthetic ones and combination of both synthetic and natural materials (e.g., PCL + gelatin and PLCL + collagen) helps to achieve sufficient hydrophilicity of scaffolds (Fu et al. 2014). In addition, several other approaches can be proposed to reduce hydrophobicity of synthetic materials, including introduction of additional functional groups to the surface and using chemical or plasma treatment, which makes them more suitable for cell attachment (Lipovka et al. 2020).

8.2.2 Biocompatibility

Optimal physicochemical properties ensure good biocompatibility of scaffold, which can be described by increased cell proliferation, adherence to the scaffold, ability to stimulate chondrogenesis and assure cell–cell interactions, and degradation at certain time points (Fig. 8.3).

8.2.2.1 Ability to Stimulate Chondrogenesis

Numerous studies showed that mimicry of physical and biological structure of cartilage might effectively direct MSCs and chondrocytes to chondrogenic lineage: increase expression of chondrogenic genes, increase expression of GAGs, and ECM

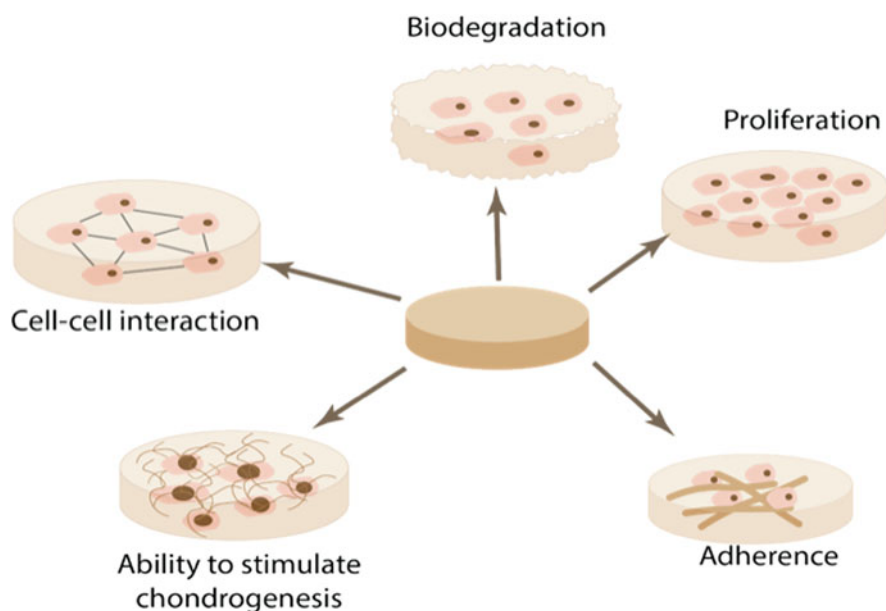


Fig. 8.3 Main properties of biocompatibility of scaffold

proteins comparing to cultivation in monolayer (Rowland et al. 2013; Tamaddon et al. 2017). Few examples are listed here, while more examples are given in Sect. 8.3: Biomimetic materials for cartilage tissue restoration. Natural polymers gelatin and chondroitin sulfate were used for scaffold formulation and it was shown that scaffold increased chondrogenic differentiation of bone marrow MSCs (BMMSCs) by increasing expression of Col II and SOX9 expression (Rowland et al. 2013). Silk and Col II scaffolds increased SOX9, Col II, Col X, and aggrecan mRNA expression on day 8 and 16 under inflammatory conditions (interleukin 1 β and tumor necrosis factor α (TNF- α) treatment) compared to synthetic PLA scaffolds (Kwon et al. 2013).

8.2.2.2 Proliferation

Biomimetic scaffold supports cell proliferation and growth within the scaffold (Dunn et al. 2006). Natural scaffolds, such as Col I, and silk scaffolds showed good biocompatibility with human adipose-derived stem cells (hADSCs) by increasing cell ability to penetrate and proliferate into the scaffold and promoting formation of the ECM on scaffold surface and pores in vitro (Barlian et al. 2018; Calabrese et al. 2017a). Numerous synthetic scaffolds also showed good biocompatibility with stem cells or chondrocytes. PLA scaffolds maintain chondrocytes' adhesion and distribution evenly on the scaffold, proliferation, and production of GAGs (Gong et al. 2008). Human MSCs proliferate and support chondrogenesis on graphene foam (GrF)/polylactic acid–poly- ϵ -caprolactone copolymer (PLC) hybrid (GrF-PLC) scaffold for 28 days thus showing a good biocompatibility (Nieto et al. 2015). As most of the studies showed good proliferation of cells on or into the scaffold, homogeneous dispersion in 3D scaffolds is hardly achieved because proliferating cells fill the pores and restrict the diffusion of water and nutrients (Dunn et al. 2006).

8.2.2.3 Adherence

Cell–substrate interaction is crucial for effective cell migration and engraftment between implanted scaffold and the host tissue (Chen et al. 2021b). Polar and positive charged surface ensures best cell attachment (Chang and Wang 2011). Most synthetic polymers are hydrophobic; therefore, ECM components are used for scaffold formulation or coating to increase adhesion while vitronectin and fibronectin naturally adsorb onto implanted scaffolds in vivo (Chang and Wang 2011). GAGs are used in scaffolds because of interactions with cells through surface receptors, which affect MSCs' behavior, adhesion, and chondrogenic differentiation (Mahsa Khatami et al. 2020; Matsiko et al. 2012). Col I application for coating has been limited because of unequal distribution and not enough penetration throughout the scaffold (Munir and Callanan 2018). It was shown that coating of chitosan fibrous scaffolds with Col II promoted MSC attachment, ECM amount, and chondrogenic differentiation of mouse bone marrow MSCs (Ragety et al. 2010). Adhesion can be increased by chemical modification of scaffold materials. HA has a high negative charge and hinders the adhesion of cells to scaffolds. Molecules of HA could be chemically modified, e.g., combined with positively charged polycations such as chitosan to improve HA scaffold features and cell adhesion potential

(Correia et al. 2011; Mahsa Khatami et al. 2020). Therefore, cell–substrate adhesion is ensured using hydrophilic or ECM component-coated scaffolds.

8.2.2.4 Maintenance of Cell–Cell Interactions

Cell-to-cell interactions, including direct contact-dependent gap junctional pathway, are important for chondrogenic differentiation (Farrell et al. 2006; Gago-Fuentes et al. 2014). Optimal pore size and surface properties can assure these interactions. Cell-to-cell interactions can be confirmed by histology and immunohistochemistry (de Windt et al. 2015). It was shown that 200–250 μm pore size supported hBMSCs cell-to-cell contact, chondrogenic differentiation, and hyaline cartilaginous ECM formation in 3D printed silica hybrid scaffolds (Li et al. 2020). Articular chondrocytes and MSCs, cultivated in direct cocultures in fibrin scaffolds, showed higher GAG production and Col II deposition than in indirect cultures, making conclusion that cell–cell interaction and communication through gap junctions is crucial for chondrogenesis (de Windt et al. 2015).

8.2.2.5 Degradation

Different degradation profiles might be achieved using degradable and non-degradable polymers. Fully degradable polymers are mostly used as a more biocompatible approach, while non-degradable materials, such as titanium alloys, have better mechanical characteristics although its integration into tissue is limited (Charlton et al. 2008; Jeuken et al. 2016). Titanium and its alloys are more attractive for bone TE (Cheng et al. 2019). Biomaterials, such as hydrogels or bioceramics, are biodegradable, but mechanically weak, not mimicking cartilage mechanical properties and risking not to withstand mechanical loading (Qu et al. 2019). Not only optimal degradation profile, but also release of bioinert products is crucial for cartilage TE. Degradation of chitosan results in release of amino sugars that can be used in metabolic processes or eliminated (Qasim et al. 2017). Degradation rate must be evaluated by rate of neocartilage formation, for instance, 12 weeks is enough to start forming neocartilage in collagen scaffolds in rabbit model, so degradation of implanted scaffold is needed to make space for new tissue (Qi et al. 2020). In vitro degradation studies can be performed by chemical (hydrolysis in buffers, other aqueous solutions) or enzymatical (elastase) degradation mimicking conditions in vivo (Eglin et al. 2009; Ren et al. 2016). Natural and synthetic polymers show different degradation profile, e.g., in PCL-gelatin scaffold within 6 weeks the majority of gelatin diffused into PBS without any sign of hydrolysis of PCL fibers (Liu et al. 2014). Poly(lactic acid) (PLA) polymers degrade in 1–5 years in vivo (Fuentes-Mera et al. 2019). Degradation of chitosan (Cs), gelatin, and hyaluronic acid (HA) and graphene (GR) scaffold was evaluated by weighing dry weight before and after lysosomal degradation. Results showed that degradation ratio decreased gradually with increased graphene content (Hu et al. 2019b). Study with architected 3D hydrogel scaffolds showed that softer and fast-degrading hydrogels promoted chondrogenesis of human articular chondrocytes more efficiently in vitro (Sarem et al. 2018). Biodegradation of scaffold can be increased by embedding matrix metalloproteinase (MMP) 7 peptide substrates within synthetic polymer

backbone (Bahney et al. 2011). To sum up, scaffolds must maintain their mechanical properties and start to degrade when neocartilage begins to form, although each polymer has a different degradation profile.

In conclusion, design of biomimetic scaffolds must mimic cartilage stiffness and porosity and have a hydrophilic surface with aligned fibers, high fluid absorption, and mechanical properties imitating physiological processes of joint motion. Bio-compatibility of scaffold is assured by enhanced cell proliferation and adherence to scaffold, maintained cell–cell interaction, induced chondrogenesis, and optimal degradation of scaffold.

8.3 Biomimetic Materials for Cartilage Tissue Restoration

Cartilage tissue is rich in GAGs and collagen fibers, of which Col II is the most abundant. Positively charged collagens and negatively charged GAGs retain a large volume of water, causing a high osmolarity microenvironment and forming a fibrillar network that gives the tissue its form and protects it from impact of mechanical load on the joint. Lipids, phospholipids, glycoproteins, and non-collagenous proteins are also found in the ECM in small amounts (Sophia Fox et al. 2009).

As ECM and its architecture have a crucial role in cartilage structure and the function of cells, it is necessary to mimic the environment required for proper cell growth. Scaffolds created from different materials can assure the right environment that allows the cells to function as in native tissue (Wasyłeczko et al. 2020). The construction of biomimetic scaffolds that imitate the specific structure and environment of chondral tissue is a major challenge in the field of cartilage TE (Hu et al. 2019a).

The main scaffold biomaterials can be chosen from natural, synthetic components, as well as their combination. Most commonly used natural scaffolds are collagens, GAGs, alginate, chitosan, hyaluronan, silk fibroin (SF), decellularized matrix, cell sheets, and others, while most commonly used synthetic scaffolds are polylactic acid (PLA)/PGA, poly(ethylene glycol), poly(vinyl alcohol), self-assembling peptides.

8.3.1 Natural ECM-Based Biomimetic Constructs for Cartilage Repair

The ECM of cartilage is mostly composed of Col II and proteoglycans. Proteoglycans include 10–15% of the cartilage of which 5% protein and 95% GAGs. The major GAGs presented in articular cartilage are chondroitin sulfate (CS) hyaluronic acid (HA), keratan sulfate, dermatan sulfate, and heparan sulfate (HS) (Menezes and Arinzeh 2020). The use of natural substances for scaffold construction is also known to reduce the inflammatory immune response (Fu et al. 2021). The main substances used for scaffolds are listed below:

Collagen. Collagens are the most common proteins in the human body and are divided into 28 subtypes (Bielajew et al. 2020). The structure and function of collagen depend on the type of collagen. Collagen types I, II, III, and V, found in vertebrates, are known to be fibril-forming collagens. Col II is the main component of native cartilage. The most abundant is Col I—a component of bone, skin, knee meniscus, ear articular cartilage, and connective tissues (Bielajew et al. 2020; Grazina et al. 2018). Col II differs from Col I in structure, distribution, and effects on cells (Ragety et al. 2010). Col I is a fibril protein containing two $\alpha 1$ (COL1A1) and one $\alpha 2$ (COL1A2) chains, while Col II consists of three $\alpha 1$ (II) (COL2A1) chains (Irawan et al. 2018). Collagen improves cell growth and adhesion through ligands, which also interact with the growth factor molecules (Kim et al. 2011). Col II is known to improve chondrogenesis through integrin interactions that modulate the TGF signaling (Schneiderbauer et al. 2004).

Collagen ensures the mechanical strength and stiffness of scaffolds. Three main types of scaffolds could be formed using collagen: hydrogels, nanofibers, and sponges. Hydrogels are formed from a branched network of collagen fibers, sponges have membranous walls, and nanofibers consist of the nonwoven network of collagen fibers, mimicking structural protein fibers (Irawan et al. 2018). Collagen can be extracted from a variety of sources: porcine, bovine (Chen et al. 2011), and marine (Hoyer et al. 2014; Pustlauk et al. 2016). Collagen extracted from marine sources does not have religious and ethnic constraints and has potential industrial applications (Oliveira et al. 2018). Collagen extracted from the jellyfish was demonstrated to be similar to vertebrate Col II by homotrimers, heterotrimers, and amount of glycine (Sewing et al. 2017). Porous scaffolds fabricated from jellyfish collagen and alginate hydrogel are mimicking the main tissue components of cartilage (Pustlauk et al. 2016). Scaffolds from collagen contain natural amino acid binding sequences, which ensure the cell–scaffold interaction (Brien 2011). Synthetic materials, for comparison, have to possess these ligands additionally incorporated. Collagen scaffolds have biocompatibility and safety approvals from FDA (Irawan et al. 2018). Col I recruits progenitor cells from subchondral bone to damaged area while Col II directs cells into a chondrocytic phenotype (Buma et al. 2003). The immunogenicity of collagen scaffolds depends on structures; hydrogels showed lower immunological response than sponges or membranes (Yuan et al. 2011). Nevertheless, the long-term use of pure Col I may be limited by weak mechanical properties, shrinkage, and induction of dedifferentiation of chondrocytes (Grazina et al. 2018; Irawan et al. 2018).

Chondroitin sulfate. Chondroitin sulfate (CS) is one of the major GAG composites of cartilage ECM which consist of repeating disaccharide units of β -1,3-linked *N*-acetyl galactosamine, and β -1,4-linked D-glucuronic acid (Yang et al. 2020). CS excels by anti-inflammatory properties, biocompatibility, negative charge, and water retention of the cartilage, which is important for pressure resistance (Chen et al. 2021a; Oliveira et al. 2018). Mainly, for scientific applications, CS is derived from animal tissues such bovine, porcine, chicken, and marine cartilage (Bishnoi et al. 2016). CS is characterized by hydrophilic nature and carboxyl,

hydroxyl functional groups, which could be modified to change physicochemical properties (Kumari and Badwaik 2019). As CS has a highly water-soluble nature, high viscosity, and poor mechanical stability (Aravamudhan 2014), CS could be combined with various polymers to achieve the required stiffness and structure. A combination of CS with other compounds, such as silk fibroin (SF), could promote the repair of articular cartilage defects (Agrawal et al. 2018). As an example, scaffolds composed of silk and CS are sponge-like structures with spherical pores, approximately 100–300 μm in diameter. Human chondrocyte cultures in a silk-CS scaffold resulted in the upregulated expression levels of cartilage matrix-related genes, including SOX9, COL2A1, and ACAN (Matsiko et al. 2015). Additional types of 3D porous scaffolds with SF and CS were fabricated and improved with the chitosan (Ch) (Matsiko et al. 2012; Menezes and Arinzeh 2020), and cartilage matrix component glucosamine (Gl) (Agrawal and Pramanik 2019). Additionally, CS could be blended with other GAGs, such as collagen. Linkage of CS to Col I results in higher metabolic activity and proliferation of bovine chondrocytes than in Col I alone (van Susante et al. 2001). It has been reported that Col II combined with CS can be used to stimulate the chondrogenic differentiation of human bone marrow-derived MSCs in the absence of growth factors (Tamaddon et al. 2017). The results of chondrogenesis gene expression and ECM secretion showed that the scaffold that was cross-linked by CS and type II collagen had the most excellent effects on the chondrogenesis of MSCs (Chen et al. 2011).

Hyaluronic acid (HA) is composed of disaccharides of glucuronic acid (Kim et al. 2011) and *N*-acetylglucosamine and is one of the main components of the ECM of hyaline cartilage and plays a tremendous role in the lubrication of joints and in regulation of protein adsorption and adhesion sites for chondrocytes in ECM (Grazina et al. 2018). The main sources of HA are animal tissues, such as rooster combs, umbilical cords, skin, and synovial fluids. Furthermore, HA can be produced by different *Streptococcus* bacteria species (Oliveira et al. 2018). HA has a high density of negative charges for which HA is very hydrophilic (Grazina et al. 2018). It is known that ECM component HA enhances cell–cell interactions, modulating the pericellular matrix in the cell condensation process, which is the initial stage of chondrogenesis (Kim et al. 2011). Several studies have shown that interactions between HA and cell-surface receptor CD44 and hyaluronan-mediated motility receptor (RHAMM) have a prominent role in maintaining differentiated characteristics of chondrocytes (Khang et al. 2007; Kim et al. 2011; Mahsa Khatami et al. 2020). The presence of HA in scaffolds creates a specific environment and promotes the synthesis of cartilage ECM (Matsiko et al. 2012). The positive effect of HA scaffolds was also observed on chondrogenic differentiation of adipose-derived MSC (Yoon et al. 2011). HA also reduces the production of inflammatory chemokines, MMPs, and regulates cell motility and proliferation (Collins and Birkinshaw 2013). The use of HA has some limitations because HA itself has rapid in vivo degradation and poor mechanical properties, which could be improved by chemical modification, additional materials, and cross-linking (Chircov et al. 2018). Previous research has indicated that the

presence of acetylated hyaluronic acid in scaffolds enhanced the chondrogenesis of hADSCs. The impact of ac-HA was proven by the overexpression of SOX9, Col II genes and confirmed by GAGs staining. Therefore, ac-HA strengthened the effect of the differentiation medium (Mahsa Khatami et al. 2020).

Other GAGs. HS is used less frequently for the fabrication of scaffolds as compared with CS and HA. HS has an impact on stem cell proliferation and differentiation processes through interactions with proteins (Zhao et al. 2015). It has been reported that HS and HS proteoglycan has the potential to be used as biomimetic biomaterials (Chen et al. 2016) and hBM-MSCs cultured on Col I and HS scaffolds with the chondrogenic medium were able to differentiate to chondrocyte-like cells (Chen et al. 2016). Other less common GAGs are dermatan sulfate (DS)—a major component of connective tissue matrix, cell surface, and membranes (Lafuente-Merchan et al. 2022) and keratan sulfate, which are rarely used for the fabrication of scaffolds for cartilage mimicry and repair.

Decellularized cartilage. In addition to macromolecules, cartilage ECM consists of proteoglycan aggregates, including mainly aggrecan and small amounts of decorin, biglycan, fibromodulin, and lumican, which form about one-thirds of the dry cartilage weight. Also, small amounts of non-collagen proteins are found, including fibronectin, tenascin, chondronectin, vitronectin, thrombospondin, and matrilin (Wojdas et al. 2021). These molecules are important for cell adhesion and differentiation and are retained in the decellularized scaffolds. Decellularization reduces the risk of the immune response by removing immunogenic antigens (Beachley et al. 2018). Therefore, application of decellularized cartilage is a way of incorporating all cartilage ECM compounds into damaged joints for re-constructing cartilage tissue. These biomaterials are usually fabricated with the use of hydrogels or fibrous polymer meshes by electrospinning (Thakkar et al. 2015). It was demonstrated that decellularized ECM in combination with chitosan (dECM-CS) upregulated the genes related to ECM synthesis and did not affect viability of rat chondrocytes. Also, the local injection treatment of dECM-CS alleviated knee joint pain with significant delay in OA progression in rats (Chen et al. 2021a).

However, decellularized cartilage scaffolds have limited porosity and scaffold density and therefore require additional processes, like pulverization or freeze/thaw cycles, to fragment the matrix and increase the scaffold permeability (Kiyotake et al. 2016; Zhang et al. 2020).

The main properties of natural cartilage ECM materials used for various scaffolds are summarized in Table 8.1.

8.3.2 Natural Non-cartilage ECM-Based Biomimetic Materials for Cartilage Repair

Various naturally occurring non-cartilage ECM-based materials have great potential for use in the scaffolds regarding biodegradability and biocompatibility properties.

Table 8.1 Essential natural ECM materials used for 3D cultures

Material	Main sources	Advantages	Limitations	References
Collagen	Porcine, bovine and marine organisms	• Good biocompatibility • Biodegradable • Non-toxic • Easy fabrication • Approved by FDA • Maintain natural morphology of chondrocytes • Col I recruited progenitor cells to cartilage defect area • Can be used as bio-ink for 3D printing	• Shrinkage • Weak mechanical strength • Rapid degradation • Immunogenicity • Dedifferentiation of chondrocytes in Col I	Buma et al. (2003), Grazina et al. (2018), Irawan et al. (2018), Kim et al. (2011), Schneiderbauer et al. (2004), Zhao et al. (2021)
Chondroitin sulfate (CS)	Bovine, porcine, chicken, and marine cartilage	• Good biocompatibility • Anti-inflammatory effect • Non-toxic • Increased metabolic activity and proliferation of chondrocytes	• Poor mechanical stability • High viscosity	Chen et al. (2021a), Oliveira et al. (2018), van Susante et al. (2001)
Hyaluronic acid (HA)	Animal tissues	• Good adhesion of cells • Extracellular matrix deposition, transport of nutrients, and metabolic product release • Biodegradability • Viscoelasticity	• Rapid in vivo degradation • Poor mechanical properties • Less methods to fabricate scaffolds	Chircov et al. (2018), Grazina et al. (2018), Kim et al. (2011), Matsiko et al. (2012), Stratton et al. (2016)
Decellularized cartilage	Cartilage	• Minimal risk of immunogenic response • Native architecture is preserved	• Limited porosity and density • High degradation • High variability between donor scaffolds	Alaribe et al. (2016), Beachley et al. (2018)

Silk is a natural fibrous protein polymer composed of two proteins fibroin and sericin and is defined as an excellent biocompatible material with great mechanical properties (Altman et al. 2003). Silk proteins are produced in fiber forms by various species of spiders and silkworms. SF is the main component of silk and is a suitable material for the construction of scaffolds because it possesses good oxygen permeability and minimal inflammatory reaction in vivo (Bhardwaj and Kundu 2012; Kundu et al. 2013). SF can be used for the fabrication of different types of scaffolds including tubes, fibers, sponges, and hydrogels (Chen et al. 2021c). As a natural biopolymer, silk slowly degrades to non-toxic and neutral products like amino acids and peptides (Voga et al. 2019). However, SF lacks cell binding domains and needs to be modified or supplemented with other polymers (Chen et al. 2021c).

Alginate is a fast-gelling naturally occurring linear polysaccharide, composed of α -L-guluronic acid (G) and β -D-mannuronic acid (M) units, and commonly applied as a cell-carrying gel (Zhao et al. 2013). Alginate is forming gels in presence of divalent cations such as calcium and magnesium (Lin et al. 2009). The main sources of alginate are brown algae, *Azotobacter* and *Pseudomonas* bacteria. Alginate degradation is a slow and uncontrollable process with high molecular weight degradation products, which could be difficult to eliminate from the body. Pure alginate structure and negative charges created by alginate in scaffolds mimic the ECM environment of cartilage (Liu et al. 2022). Numerous studies have been reported that alginate supports chondrogenesis and is a promising biomaterial in cartilage regeneration. For example, purified alginate gel had favorable effects on chondrogenesis of rabbit BMMSCs. Culturing of BMMSCs in ultra-purified alginate gel beads and in complete DMEM-HG chondrogenic medium with 10 ng/mL recombinant human TGF β 3 showed upregulation of Col II, ACAN, SOX9 genes (Igarashi et al. 2010). However, naturally found alginate is hydrophilic and bioinert material, which lacks mechanical strength, badly interacts with ECM, cells and needs to be modified or supplemented with other materials, such as collagen, HA, CS, PLA, and polycaprolactone (PCL) (Farokhi et al. 2020; Irani et al. 2021).

Chitosan is a polysaccharide obtained during deacetylation of chitin (Chung and Chang 2010). Chitin is mainly extracted from the exoskeleton of shellfish, insects, and cell walls of fungi (Comblain et al. 2017). Chitosan structure is highly similar to GAGs found in ECM, resulting not only with good adherence, but also low immunogenicity. β -(1,4) Glycosidic bonds make chitosan elastic and flexible. Chitosan is biodegradable and its degradation produces only harmless amino acids and sugars making it a safe polymer for TE (Islam et al. 2020). Poor mechanical properties, quick loss of shape and size limit the application of chitosan as a biomimetic scaffold (Tan et al. 2009). Chitosan properties depend on the molecular weight of the monomer, calcium content, and the degree of acetylation, which could be modified according to needs (Ravanetti et al. 2015). It has been reported that the addition of raffinose to chitosan scaffolds makes construction smoother and elastic (Bettini et al. 2008).

Gelatin is obtained by thermal denaturation or hydrolysis of collagen and consists of arginine-glycine-aspartic acid (RGD) sequences, which are important for cell attachment (Yue et al. 2015). Properties of gelatin depend on the type and source of collagen (Bonani et al. 2018). Biodegradability, biocompatibility, and mechanical properties of gelatin are suitable for mimicry of cartilage tissue (Li et al. 2018). However, the use of pure gelatin as scaffold is limited because of instability at temperatures higher than 35 °C (Bonani et al. 2018), poor mechanical properties, and fast enzymatic degradation (Afewerki et al. 2019). However, gelatin can be modified or used in combination with other polymers to improve mechanical properties of scaffolds (Bonani et al. 2018). Gelatin methacrylamide (GelMA) is the most widely used chemical modification of gelatin for TE (Li et al. 2019) which forms cross-linked hydrogels under ultraviolet light (Suo et al. 2020). GelMA hydrogels are widely used for cartilage regeneration because they have good biocompatibility and adjustable mechanical properties, which are important for cell adhesion, proliferation, and differentiation (Piao et al. 2021). In most cases, GelMA needs to be supplemented with additional synthetic and natural materials to improve biological and mechanical features, required for cell behavior (Klotz et al. 2016).

Bioceramics are widely used as coatings on metal joint prostheses, powders, and granules for bone filling, bone cement, and porous scaffolds. Bioceramics have a potential to be used as scaffolds because they can create stable bonds with the host tissue. Furthermore, ceramics are bioactive, bioresorbable, and degrade gradually, while being replaced by the natural host tissue (Baino et al. 2015). The main types of bioceramics are hydroxyapatite (HAP), bioactive glass, and calcium phosphate or sulfate (Köse et al. 2018). The use of bioceramics for cartilage biomimicry has some limitations such as poor elasticity and high stiffness (Deng et al. 2019).

Hydroxyapatite (HAP) ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) is a calcium phosphate ceramic—the main inorganic component of teeth and bones (Gonzalez and Alvarez 2014). HAP is commonly used as granules and scaffolds for bone tissue regeneration. HAP can be synthesized or extracted from natural sources like fish bones, eggshells, shrimp shells, and bovine bones (Ramesh et al. 2017). Properties and applications of HAP depend on chemical composition, size, and crystallinity. HAP in nanoscale exhibits better bioactivity than in microscale sizes (Lin and Chang 2015). HAP increases the compressive strength and the elasticity of the scaffolds (Szcześ et al. 2017) and creates a porous structure, which allows sufficient integration with the host tissue (Luo et al. 2020). According to the early studies, culturing chondrocytes with HAP promotes the formation of mineralized cartilage (Kandel et al. 1997). However, required properties of scaffolds could be achieved with the supplementation of HAP with other materials. It has been reported that nano-HAP/PVA (poly(vinyl alcohol)) gel composite properties are similar to natural articular cartilage. The response of gel composites to external mechanical stress was similar to articular cartilage (Yusong et al. 2007). PGA scaffolds supplemented with HAP stimulated the adhesion and proliferation of porcine chondrocytes in comparison with pure PGA scaffolds (Lee et al. 2008).

Bioactive glass consists of many types which can be obtained by using different techniques. Bioactive glass has an amorphous structure and good cell affinity and can release various bioactive and cell stimulatory ions (Na^+ , Ca^{2+}) during the degradation process (Gögele et al. 2021; Sergi et al. 2020). The most widely used is silicate glass, which is known by its commercial name Bioglass[®] or 45S5. This type of glass releases soluble silica, has high surface area and nanometer-scale porosity, and rapidly forms hydrated silica gel (Hench 2006). It has been reported that silicate bioglass is used for cartilage repair. Bioglass can be added to natural polymers to improve physical and mechanical properties. Bioglass is usually combined with proteins (collagen, gelatin), polysaccharides (HA, Ch, alginate), and other polymers (Rahaman et al. 2011). Biodegradable, biocompatible hydrophobic polyester poly(hydroxybutyrate-*co*-hydroxyvalerate) (PHBV) in combination with bioglass showed great potential for use as chondrocyte carriers. With the addition of bioglass to PHBV, hydrophilicity and mechanical strength of scaffolds was improved. Water contact angle decreased from 65° to 32°. Rabbit chondrocytes subcultured with scaffolds maintained their chondroblast-like phenotype and produced ECM. It was evaluated that the amount of collagen, GAG, and cartilage oligomeric matrix protein was significantly higher in the PHBV scaffolds with bioglass compared to PHBV alone (Wu et al. 2013). However, the 45S5 type of bioglass has some limitations, such as difficult processing into porous 3D scaffolds (Rahaman et al. 2011). Fragility, surface instability, and difficult processing to 3D scaffolds are disadvantages limiting the use of bioactive glass in scaffolds (Lin et al. 2020).

Examples of different natural non-cartilage ECM-based materials and their main properties are summarized in Table 8.2.

8.3.3 Synthetic Materials for Cartilage Repair

Synthetic polymers are attractive scaffold materials because they have an easy processing capacity and can be modified to achieve the desired properties (Dwivedi et al. 2020). Polymers such as polyurethane (PU), PCL, poly(lactic acid) and PGA are widely used for scaffold development owing to their mechanical properties and processability. Degradation kinetic of synthetic materials depends on chemical composition and environment and varies from weeks to years (Sergi et al. 2020).

Hydrophilicity of materials plays a crucial role in regulation of cell adhesion and growth on the scaffold surface (Wu et al. 2013). Synthetic polymers could be classified as hydrophilic materials (bioactive glass), and hydrophobic materials PGA, poly(lactic acid) (PLA), poly(ϵ -caprolactone) (PCL). Many polymers such as PGA, PLA, and PCL are already clinically used as biomaterials. The use of those polyesters has some limitations because they are hydrophobic and have poor wetting and cell attachment properties. Furthermore, these polymers degrade to acidic products, which can cause undesirable responses (Janik and Marzec 2015).

Table 8.2 Main natural non-cartilage ECM-based materials

Material	Main sources	Advantages	Limitations	References
Silk	Spiders, silkworms	• Biocompatibility • Oxygen permeability • Safe degradation products • Great mechanical properties (breaking strength, modulus, and elongation) • Large-scale processing lowered the cost • Approval of FDA for soft tissue repair • Limited bacterial adherence	• Lack of binding domains • Immunogenicity which can be reduced by chemical modifications • Degradation can be controlled from weeks to years	Bhardwaj and Kundu (2012), Bhattacharjee et al. (2017), Chen et al. (2021c), Kundu et al. (2013), Mottaghitalab et al. (2015), Voga et al. (2019), Widhe et al. (2012)
Alginate	Brown algae, <i>Azotobacter</i> and <i>Pseudomonas</i> bacteria	• Biocompatibility • Negative charge • Approved by the FDA as wound dressing material • Good chondrogenic potential	• Slow, uncontrollable degradation in vivo • Low cell attachment • Uneven distribution of cells	Farokhi et al. (2020), Igarashi et al. (2010), Irani et al. (2021), Liu et al. (2022), Widhe et al. (2012)
Chitosan	Deacetylation of chitin	• Hydrophilicity promotes cell adhesion, proliferation, differentiation • Structure is similar to GAG • Low immunogenicity • Slower degradation than other natural polymers • Safe degradation	• Poor mechanical properties • Quick loss of shape and size	Islam et al. (2020), Lee et al. (2017), Tan et al. (2009), Zhao et al. (2021)

(continued)

Table 8.2 (continued)

Material	Main sources	Advantages	Limitations	References
		products, elastic • Easy immobilization of DNA allows gene delivery		
Gelatin	Synthesized from partial hydrolysis of animal collagen	• Very close molecular structure and function to collagen • Biocompatibility • Low cost • Good cell attachment	• Poor mechanical properties • Low thermal and enzymatic stability • Risk of immune response	Bonani et al. (2018), Li et al. (2019)
Bioceramics (HAP, bioactive glass)	Fish bones, eggshells, shrimp shells, bovine bones, corals	• Good cell affinity • Improves physical and mechanical properties of scaffolds	• Fragility • Surface instability • Difficult processing to scaffolds • Promotes formation of mineralized cartilage	Deng et al. (2019), Diaz-Rodriguez et al. (2019), Hench (2006), Kandel et al. (1997), Szcześ et al. (2017)

PCL is semi-crystalline linear aliphatic polyester, defined as a material with good physical–chemical and mechanical characteristics, such as easy processability, non-toxic degradation products, and safety for biomedical applications (Neves et al. 2011). The degradation time of PCL is 2–3 years, which is slower than other polyesters because of the presence of five hydrophobic $-\text{CH}_2$ moieties. PCL is widely used for scaffolds in regenerative medicine and drug delivery applications (Dwivedi et al. 2020). PCL addition to the scaffolds leads to a higher fiber surface roughness, increase of compressive mechanical properties, and decrease of swelling ratio (Neves et al. 2011). However, it has some limitations, such as low cell attachment and slow degradation (Ishaug-Riley et al. 1999). PLC is usually combined with other materials to reduce its hydrophobicity (Fu et al. 2016).

Poly(Lactic Acid) (PLA) is a thermoplastic polymer, a poly α -hydroxy ester with helix structure, commonly used for scaffolds in orthopedics and dental applications because of its biocompatibility, strength, and stiffness properties (Gomez-Sanchez et al. 2014; Narayanan et al. 2016). Additionally, PLA is an attractive material for various implants because of degradation via hydrolysis and product elimination through cell metabolism (Santoro et al. 2016). Lactic acid (2-hydroxypropionic acid) is the monomer of PLA (Davachi and Kaffashi 2015). During the PLA synthesis, the stereoregularity of this monomer determines the

physicochemical properties of the material (Santoro et al. 2016). From the literature, it is known that PLA-based scaffolds are suitable for cartilage TE (Narayanan et al. 2016). However, PLA use for scaffolds has some limitations as low cell adhesion to the surface (Yagi et al. 2021). To improve cell attachment and spreading to PLA, surface modification may potentially be used to create materials that stimulate cell adhesion and maintain differentiated phenotype (Chen et al. 2010).

PGA chemical structure is similar to PLA but missing the methyl side group. Polymer chains of PGA are packed together tightly and have a high degree of crystallinity (Samantaray et al. 2020). PGA has excellent biocompatibility, high thermostability (Budak et al. 2020), and affinity for chondrocytes. PGA degrades via chemical hydrolysis, rather than enzymatic hydrolysis (Itani et al. 2014). However, degradation of PGA is faster than PLA (Budak et al. 2020). Scaffolds from PGA have a small range of mechanical properties, a hydrophobic surface that is not beneficial for attachment and proliferation and could be overcome by using other materials (Moran et al. 2003).

PU is used to synthesize shape memory materials. PU has a unique segmented structure, due to which various properties can be obtained using different materials. Synthesis techniques and composition affect the mechanical and physical properties of PU (Janik and Marzec 2015). PU has high elasticity, good biocompatibility, tensile strengths and is widely used in medical devices (Wen et al. 2019). The degradation of PU could be hydrolytic, enzymatic, or oxidative and depends on environmental conditions, size, molecular weight, and composition (Tsai et al. 2015). PU has negative charge, which makes it sensitive to the environment (Tsai et al. 2015). Scaffolds prepared only from linear PU had some limitations. Biomechanical properties of the cell-polymer scaffold constructs differed from those of the natural cartilage. The content of GAGs and total collagen in native bovine articular cartilage was significantly higher as compared with young calf's fetlock chondrocytes cultured in polymer constructs for 42 days. Adherence of chondrocytes to PU scaffolds was limited (Grad et al. 2003). Nevertheless, the way of synthesis and composition of PU may help to avoid mentioned limitations. PU scaffolds synthesized by freeze-drying had excellent pore interconnectivity and tensile properties. In addition, evaluating chondrogenesis of human BMMSCs in PU scaffolds and comparing with PLA, the expression of chondrogenic genes (SOX9 and ACAN) in PU scaffolds was upregulated (Tsai et al. 2015).

PVA—polyvinyl alcohol—is a synthetic polymer derived from polyvinyl acetate through hydroxylation. The physical, chemical, and mechanical properties of the PVA depend on the amount of hydroxylation. The higher the degree of hydroxylation—the lower the solubility in water (Baker et al. 2012). PVA is the most popular synthetic water-soluble, non-toxic, biocompatible, and biodegradable polymer. PVA hydrogels are used for regeneration of cartilage due to their high water content, high elastic and compressive mechanical properties. Main limitations of pure PVA for cartilage are low mechanical strength and poor lubricity. Different modifications and polymers could be used to reduce the

limiting properties of PVA. Biopolymers such as cellulose, chitosan, various fibers, PEG, PCL, and collagen could be combined with PVA (Chen et al. 2021b).

PLGA—poly lactic-*co*-glycolic acid is a biocompatible and biodegradable material. Main biomedical applications of PLGA, such as drug delivery and tissue engineering, are approved by FDA. It has been reported that PLGA microspheres could be used for cartilage tissue engineering (Qu et al. 2021). It has been reported that PLGA improves thickness and mechanical properties of cartilage, formed in vitro with porcine articular chondrocytes (Zhang et al. 2012). Furthermore, PLGA scaffolds have a positive effect on chondrocyte proliferation and ECM formation in vitro. However, PLGA scaffolds have some disadvantages, such as poor hydrophilicity and weak absorbance of proteins (Zhao et al. 2016).

Poly(L-lactide-*co*- ϵ -caprolactone) (PLCL). PLCL is biocompatible, biodegradable, and elastic polymer, made of PLA and PCL. PLCL is hydrophobic and lacks cell recognition sites (Zhou et al. 2020). The elastic moduli of PLCL is easily regulated by changing the content of PLC (Liu et al. 2020). PLCL scaffolds could be used for cartilaginous tissue formation. As an example, it has been reported that rabbit knee joint chondrocytes cultured on elastic PLCL scaffolds properly proliferated and differentiated in vitro (Jung et al. 2010). Furthermore, chondrocyte-PLCL constructs implanted in mice for 5 weeks formed well-developed cartilaginous tissue (Jung et al. 2008a).

Graphene oxide (GO). Graphene is a crystalline form of carbon. GO is graphene derivative and is characterized as an amphiphilic material because it contains both hydrophobic and hydrophilic regions (Qu et al. 2018). It is known that GO is one of the thinnest materials and is widely used for tissue engineering. GO has large surface area, good electrical conductivity, high tensile strength (Shamekhi et al. 2019) and is often used to increase the biomechanical strength of other materials (Gong et al. 2021). The presence of GO in scaffolds can stimulate the chondrogenic differentiation of cells. Furthermore, GO composites are able to be functionalized with fibronectin and TGF- β proteins through π - π and electrostatic interaction (Shin et al. 2016). GO could be combined with natural materials, such as chitosan (Shamekhi et al. 2019), alginate (Olate-Moya et al. 2020) to improve their mechanical, physical, and electrical properties. It has been reported that the GO-chitosan complex and its surface roughness stimulated human articular chondrocyte proliferation (Shamekhi et al. 2019).

Poly(ethylene glycol) (PEG). PEG is characterized as a biodegradable and non-toxic material with a highly soluble nature and good mechanical properties (Ngadimin et al. 2021). PEG is a polyether extensively used as supporting material in cartilage research studies (Duarte Campos et al. 2012). Application of this material attracts attention because it cannot be recognized by the immune system and is rapidly eliminated from the body through liver or kidneys (Zhao et al. 2021). The diversity of PEG in molecular weight and chemical structure makes it possible to prepare scaffolds with required mechanical properties (Gao et al. 2017; Kim et al. 2021).

Polyethersulfone (PES). Usually, PES material is used for hemodialysis, filtration, and ultrafiltration (Mahboudi et al. 2018a). PES scaffolds for cartilage repair are

used less frequently than scaffolds from other polymers. It has been reported that chondrogenesis of human iPSCs cultured on PES scaffolds was better as compared with scaffold-free method. Expression of cartilage-specific genes collagen type II, collagen type X, and ACAN was upregulated (Mahboudi et al. 2018b). Another study showed very similar results. PES scaffolds enhance chondrogenic differentiation of human BMMSCs, evidenced by higher expression of ACAN and collagen II genes (Mahboudi et al. 2018a).

The main properties of widely used synthetic materials for cartilage repair are listed in Table 8.3.

In summary, synthetic polymers have some disadvantages. Poor biocompatibility properties limit the use of synthetic polymers (Kim et al. 2011). Furthermore, long degradation period, undesirable degradation products, and poor cell attachment properties (Shen et al. 2018) need to be modified by coating or adding adhesion peptides or proteins, or used in combination with natural bioactive materials (Kim et al. 2011).

8.3.4 Mixed Biomimetic Materials for Cartilage Repair

Mixed scaffolds of natural and synthetic materials are of great interest in TE as they ensure both physical and biological properties of the construct, resembling the tissue's natural environment and being mechanically stable (Lin et al. 2019; Neves et al. 2020). The superiority of combined or hybrid constructs over non-hybrid ones for cartilage tissue regeneration includes both integration of the construct to local tissues and induction of cell differentiation and their growth (Sánchez-Téllez et al. 2017).

A number of in vitro studies have demonstrated a strong chondrogenic protein synthesis and gene expression response of both stem cells and chondrocytes seeded or incorporated into mixed scaffolds. For instance, chondrogenic differentiation of adipose tissue derived stem cells, incorporated into chitosan/gelatin hybrid hydrogel scaffold, was more pronounced according to histochemical evaluation as compared to cell differentiation without scaffolds (Song et al. 2015). Chitosan/sodium β -glycerophosphate/Gelatin (Cs/GP/Gel) hybrid scaffold has also stimulated chondrogenesis of BMMSCs by synthesizing large amounts of GAGs (Hu et al. 2019a). Moreover, hybrid scaffold comprising of two layers of hyaluronic acid hydrogel PLA nanofibers, arranged in a specific order (corresponding to the three zones of native cartilage fiber orientation) was inoculated with primary bovine chondrocytes and synthesized different amounts of Col I, Col II and GAGs in ECM (Owida et al. 2018). Silica/poly(tetrahydrofuran)/poly(ϵ -caprolactone) hybrid scaffolds with different pore sizes were also introduced for human BMMSCs. Small pores induced collagen II synthesis, while larger pores resulted in cartilage I biosynthesis, which is typical for fibrocartilage (Li et al. 2020). Sodium alginate/polyglutamic acid and microcrystalline cellulose hybrid construct have shown mechanical strength, biocompatibility, and induced chondrogenic gene expression

Table 8.3 Synthetic materials for cartilage repair

Material	Advantages	Limitations	Reference
PCL	• Good cell attachment, proliferation, ECM production • Easy processability of different 3D structures • Non-toxic degradation products • Mechanical stability • Approved by FDA for use in humans • Compatibility with wide range of polymers • Maintained natural chondrocyte phenotype • Relatively low cost	• Poor cell adhesion because of hydrophobicity • Slow degradation	Azimi et al. (2014), Jeong and Hollister (2010), Zhao et al. (2021)
PLA	• Good cell attachment, proliferation, production of ECM • Mechanical stability • The absence of peptide linkages minimizes immunogenicity • FDA approval • Can be easily blended with other polymers • Faster degradation than PCL	• Poor water absorption • Poor cell adhesion to the surface • Release of acidic products during degradation	Chu et al. (1995), Haaparanta et al. (2014), Rosenzweig et al. (2015), Zhao et al. (2021)
PGA	• Thermal stability • Approved by FDA • Good biodegradability • Lack of toxicity • Low immunogenicity	• Release of acidic products during degradation	Zhao et al. (2021)
PU	• Elasticity is similar to ECM • Degradation in vivo • More hydrophilic than PLA • Better mechanical properties (elongation, compression recovery) than PLA • Good cell attachment and ECM production	• Sensitivity to environmental conditions • Aromatic diisocyanate-based PU could be potentially toxic • Dedifferentiation of chondrocytes during prolonged cultivations	Grad et al. (2003), Tsai et al. (2015)
PLGA	• Approved by FDA • More hydrophilic than PLA, PCL • Better cell attachment than PLA	• Mechanical properties slightly lower than cartilage • Cells do not	Caminal et al. (2016), Ouyang et al. (2002), Shin et al. (2006), Uematsu et al. (2005), Zheng et al. (2011)

(continued)

Table 8.3 (continued)

Material	Advantages	Limitations	Reference
	- The rate of degradation can be regulated - Promoted cell migration, attachment, proliferation, cartilage regeneration	migrate deeply into the scaffold	
PLCL	- High elasticity and recovery from strain - Higher ECM production than PLA - Mechano-active - Degradable - Maintain natural morphology of chondrocytes	- Dedifferentiation of cells - Insufficient to create 3D networks	Jung et al. (2012), Jung et al. (2008b), Kim et al. (2015), Xie et al. (2006)
PVA	- Non-toxic - Biodegradable - High elastic and compressive mechanical properties	- Low mechanical strength - Poor lubricity	Baker et al. (2012), Chen et al. (2021b)
PEG	- Non-toxic - Good mechanical properties - Approved for clinical use by the FDA - High cytocompatibility	Low cell adhesion due to hydrophilicity	Fu et al. (2016), Zhao et al. (2021)
PES	- Thermal and chemical resistance - Increased chondrogenic differentiation - Hydrolytic stability	- Hydrophobicity - Unmodified surface has low cell attraction - Nonbiodegradable	Abedin Dargoush et al. (2020), Mahboudi et al. (2018a, b), Shabani et al. (2011)
GO	- Thin - Good electrical conductivity - Large surface area - High tensile strength - Amphiphilicity	Risk of immune response	Geetha Bai et al. (2019), Qu et al. (2018), Raslan et al. (2020)

of stem cells (Wang et al. 2022). Porous hybrid PCL/Col I scaffolds with varying concentration of collagen I were produced by thermally induced phase separation. The size of pores depended on Col I and PCL concentration (Munir and Callanan 2018).

In vivo studies were less examined with hybrid constructs. However, the results are promising. PU foam coated under vacuum with calcium phosphates (PU/VAC) scaffolds combined with human amniotic MSCs have shown to stimulate

chondrogenic differentiation and form cartilaginous tissue, when implanted to the mechanically created defect in the rabbit medial femoral condyle, without causing any inflammation or immune response (Tschon et al. 2020). Also, methacrylated gelatin (mGL) hydrogel and methacrylated HA combined scaffold stimulates chondrogenesis and subchondral bone regeneration after 12 weeks of scaffold implantation (Kwan et al. 2020). 3D printed scaffolds, such as PCL/SF hybrid scaffold, were also used for meniscus regeneration. Synovium-derived MSC-specific affinity peptide was conjugated with these scaffolds to induce adherence of synovium-derived MSC in vivo in rabbit damaged meniscus. Histological, biochemical, and biomechanical properties of the newly formed tissue in the disturbed meniscal area after 24 weeks of scaffold implantation have demonstrated high resemblance to native meniscus (Zhang et al. 2017). Another meniscal study demonstrated promising results with PCL and sodium chloride 3D printed hybrid scaffold for cartilage tissue regeneration. Seeded with autologous fibrochondrocytes constructs were transplanted to the rabbit destroyed knee cartilage and after 12 week of this implantation properties of neo-meniscus were measured immunohistochemically and microscopically and proved that new meniscus resembles native one (Jeong et al. 2021).

These findings have provided novel aspects regarding scaffold application for both in vitro and in vivo studies. However, one material cannot fully mimic physical and biomechanical characteristics of natural cartilage, so scaffolds composed of both natural and synthetic materials are promising for future TE therapies.

8.4 The Effects of External Physical and Chemical Stimuli on ECM-Based Scaffolds/Structures

To increase chondrogenesis, external physical and chemical stimuli can be applied on biomimetic scaffolds with chondrocytes or MSCs. Chemical stimulus is induced by encapsulation of growth factors to maintain prolonged stimulation of differentiation.

Various growth factors and other molecules are added to chondrogenic induction medium to mimic the natural cartilage healing process. Recent studies show that growth factors can be incorporated in scaffolds by different methods to assure prolonged action from a few days to a few months. Growth factors can be loaded directly, encapsulated, attached via covalent bond and reversibly bound (Madry et al. 2014). These molecules include TGF- β 1/3, BMP family proteins, insulin-like growth factor (IGF), kartogenin (KGN), etc.

TGF- β 1 was attached to scaffold via affinity interactions with alginate sulfate, resulting in sustained release of TGF- β 1 for more than 7 days in vitro. It promoted chondrogenic differentiation of human MSCs by inducing condensation and expression of chondrogenic biomarkers (Col II, aggrecan) (Re'em et al. 2012). BMP-7/TGF- β 3 were encapsulated with heparin and Tetronic 1107 forming nanocomplexes in L-lactide-co-glycolide copolymer (PLGA) scaffolds. Both protein sustained release was provided for at least 30 days increasing expression of chondrogenic

biomarkers in human MSCs even in the absence of other 691 external differentiation factors (Crecente-Campo et al. 2017). Rabbit BMMSCs were seeded on decellularized porcine articular cartilage scaffold with PLGA microspheres (MP), slowly releasing KGN. Scaffold was biocompatible and promoted BMMSCs proliferation. Histological analysis demonstrated an increased amount of Col II and GAGs proving induced chondrogenic differentiation (Zhao et al. 2020a, b). IGF-1 loaded into PLGA nanoparticles and incorporated onto 3D printed PCL scaffolds increased chondrocyte proliferation, GAGs content, expression of SOX9, Col II, ACAN, and HIF-1 alpha gene expression making it a promising candidate for further studies (Wei et al. 2020). Scaffolds can not only deliver growth factor proteins, but also they may be used as a vehicle gene. Col II-glycosaminoglycan scaffold was used as a non-viral IGF-1 plasmid vehicle for chondrocytes. The sustained release of IGF-1 increased tissue formation, chondrocyte-like cells, levels of GAGs and Col II (Capito and Spector 2007). Therefore, scaffolds can be not only passive support for cells, but also a source of growth factors, stimulating chondrogenesis.

Taken together, although scaffolds themselves modulate cellular processes and behavior, chemical stimuli, mimicking natural healing, might enhance chondrogenesis and show more promising results.

8.5 Application of Scaffolds for In Vivo Studies

In vitro conditions cannot fully mimic physiological processes, biocompatibility of scaffold in vivo requires low immunogenicity and high integration, degradation involves complex of different chemical and biological reactions occurring at the same time and all of these processes also affect the success of chondrogenesis.

Host cell migration into scaffold is a critical step ensuring integration of scaffold into host tissue. It is known that naturally chondrocytes are surrounded by dense layers of ECM which limits migration of chondrocytes into scaffolds. Cell migration can be evaluated by scanning electron microscopy (SEM) and histological analysis (Pabbruwe et al. 2009). Cell-free gelatin methacrylate/ECM—functional peptide sequence PFSSTKT (GelMA/ECM-PFS) scaffolds showed a good integration after implantation into rabbit knee cartilage with microfracture by recruiting endogenous stem cells and formed neocartilage with morphology and mechanical properties similar to normal hyaline cartilage (Huang et al. 2022). Cell-free Col I scaffold was planted subcutaneously in mice where it promoted recruitment of host cells and augmentation of the cartilage-like tissue (Calabrese et al. 2017b). Apoptosis of chondrocytes at the edge of wound after surgery limits integration of scaffold and it can be reduced by enzymatic matrix digestion (Pabbruwe et al. 2009).

Rejection of scaffold by host organisms is one of the main issues concerning scaffold biocompatibility. It limits application of natural cartilage while natural or synthetic ECM with lower antigenicity and higher biocompatibility are more preferred for TE (Gupta et al. 2014; Huang et al. 2022). The efficacy of decellularization and purity of the biomaterials must be appropriately identified to avoid potential post-implantation activation of the immune response. Poly(L-Glutamic acid)-graft-

tyramine (PLG-G-TA) hydrogels showed an acceptable biocompatibility without causing tissue edema and necrosis after 4 weeks of subcutaneous implantation, the level of inflammation decreased along with degradation of scaffold (Ren et al. 2016).

In vivo degradation has a more complex profile, including not only chemical or enzymatic degradation, but also degradation by immune cells, reactive species (Ren et al. 2016). It is commonly known that natural scaffolds have higher degradation rate in vivo, e.g., chitosan and Col II scaffolds implanted in rats are almost completely degraded within 8 weeks. Degradation of porous chitosan HAP fabricated via freeze gelation was evaluated in chinchilla rabbits. Chitosan membrane fully degraded by day 30 while HAP reduced degradation rate of CH-based scaffolds, making them more attractive for hard TE (Huang et al. 2013). Although natural polymers have higher degradation rate, different amounts of gelatin did not have any effect on degradation of PLG-g-TA hydrogels in the subcutaneous layer of rats. Products of degradation were fully phagocytized and metabolized without causing inflammation and making it more biocompatible than synthetic materials (Ren et al. 2016). Synthetic scaffolds have lower degradation rate, depending on physical qualities, for instance PLGA has more hydrophilic side chains making it more degradable than PCL (Huang et al. 2013). Degradation of synthetic scaffolds can be modified by varying chain extension and cross-linking. Short (4 months)- and long-term (13 months) degradation profiles of PCL copolymer-based polyester polyurethane-urea (PSPU-U) scaffolds were evaluated in rat model to determine the optimal degradation time for cartilage repair. Results showed that short-term degradation increased amount of hyaline-like tissue, amount of Col II, volume filling of cartilage defect and had a better qualitative cartilage repair after 8 and 16 weeks of implantation as compared to long-term (Shah et al. 2017).

Methacrylated gelatin/methacrylated hyaluronic acid (mGL/mHA) (9:1) hybrid cell-free scaffold was used on the defect site of rabbit cartilage. After 12 weeks, histological analysis with Safranin-O showed a positive staining of the mGL/mHA group, although it was lower than normal cartilage. High amount of cells was observed within the defect site (Lin et al. 2019). 3D hydrogel scaffolds, produced by cross-linking gelatin with ethyl lysine diisocyanate (LDI) with human articular chondrocytes, were implanted in an ectopic site of 6–8-week-old female nude mice for four weeks. Results negated in vitro data and showed that stiffer and slow degrading hydrogels increased chondrogenesis and cartilage formation more efficiently than soft ones (Sarem et al. 2018). Nanofibrous PCL/gelatin scaffolds with iPSC cells were implanted on rabbit cartilage defect, the results of cartilage healing were evaluated after 6 weeks and 3 months. It was shown that cell seeded scaffold enhanced gross appearance; histological and immunohistochemical analysis showed increased level of cartilaginous matrix and Col II, Western blot showed increased Col II, aggrecan, and SOX9 expression. Rabbits were under immunosuppression, so biocompatibility was not evaluated (Liu et al. 2014).

These studies underline the difficulties in investigation of biomimetic scaffolds. While improved chondrogenesis was shown promisingly, full integration of scaffold into host tissue is limited by immunogenicity or not enough biocompatibility.

8.6 Conclusions

Biomimicry in biomedical research aims to imitate native environment seeking to recapitulate natural processes and structures of tissues, organs, etc. For cartilage TE, scaffolds can be constructed using different natural and synthetic materials that mimic the properties of native tissue. By combining a number of technologies, it is possible to improve biomimicry. Natural or synthetic scaffolds, mimicking cartilage physicochemical properties can successfully enhance chondrogenesis in vitro and in vivo. These physicochemical properties include stiffness, surface properties, porosity, fluid absorption, electrical conductance, and piezoelectric properties with or without external stimuli. The inclusion of chemical stimuli imitates physiological processes, such as release of growth factors. However, challenges remain, including simulation of stiffness, creating porosity gradient, optimal fiber alignment, homogeneous dispersion of cells, and interchanging growth factor release. Therefore, further studies are needed to achieve satisfactory mimicry.

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Cartilage Tissue Engineering: Advances and Frontiers

9

Mahsa Fallah Tafti and Shahab Faghihi

Abstract

Defects in the cartilage caused by trauma, osteoarthritis, or other disorders invariably result in severe joint pain and dysfunction. Without access to progenitor cells, blood, and nutrition, the damaged cartilage would be incapable of self-repair. While current clinical treatments such as autogenous and allograft osteochondral transplantation techniques have demonstrated some efficacy, there are limitations such as donor insufficiency and poor integration with adjacent tissue. Advanced scaffold-based versus scaffold-free classifications for replicating cartilage's native architecture and functional properties are the most recent consideration in cartilage regeneration. This chapter underlines the latest progress in cartilage tissue engineering techniques. Plus, the emerging technologies, such as 6D printing for generation of customized cartilage implants which can be used to mitigate or model disease states in clinical studies, are highlighted.

Keyword

Cartilage · Tissue engineering · Customized model · Regenerative medicine

M. Fallah Tafti · S. Faghihi (✉)

Stem Cell and Regenerative Medicine Group, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

e-mail: sfaghihi@nigeb.ac.ir

9.1 Introduction

Cartilage is a connective and heterogeneous tissue with avascular, aneural, and alymphatic features. It is a complex tissue with multiple roles in the body including bearing loads in intervertebral disks, articular joints as well as providing joint lubrication (Krishnan and Grodzinsky 2018; Mobasheri et al. 2014). Hyaline cartilage, fibrocartilage, and elastic cartilage are majorly three types of cartilage with high collagen content (Daly et al. 2016; Barbon et al. 2021). There are many extracellular matrix (ECM) components and a small number of chondrocytes in native cartilage. Chondrocytes are vital in producing the ECM during growth and maintaining the tissue during adult life (Daly et al. 2016; Agarwal et al. 2021). Chondrocytes also provide sustained nutrient and gas diffusion over large distances (Agarwal et al. 2021). Nowadays, cartilage-associated diseases include osteoarthritis, trachea stenosis, chondrocalcinosis, trauma, tumor resection, malformation, inflammation, and degeneration (Shen et al. 2019). Lack of blood circulation and innervation can limit self-regeneration and intrinsic repair of cartilage (Huey et al. 2012). Moreover, there are no effective drugs and treatments for defected cartilage (Krishnan and Grodzinsky 2018). Medical interventions such as multiple drilling, abrasion arthroplasty, microfracture, mosaicplasty (autologous osteochondral grafts), and cellular transplantation (autogenous and allogeneic chondrocytes) for cartilage defects are current cartilage repair techniques (Kwon et al. 2019; Farr et al. 2011). However, the functional regenerative repair of cartilage injuries remains a scientific and medical challenge (Bakhshayesh et al. 2019). For instance, allografts can lead to the transmission of diseases or provoke immunologic responses in the recipient, and once in place, they remodel very slowly and patients are required to undergo several surgeries (Gomoll and Minas 2014). Alternative therapeutic methods include tissue engineering using cells, growth factors, and biomaterials to fabricate biological and functional substitutes to replace damaged tissues (Lee et al. 2018). Current tissue engineering strategies struggle in the attempt to create applicable constructs in cartilage regeneration. Conventional fabrication techniques include phase separation, gas foaming, particle/salt leaching, solvent casting, and freeze-drying. These methods on different physical principles have been used to fabricate biological constructs in cartilage tissue engineering (Zhao et al. 2018; Moroni et al. 2018; Donate et al. 2020). Electrospinning can also be employed to prepare controllable nanofibers which can accurately mimic ECM structures (such as fibrous collagen) (Wang et al. 2013). The electrospun scaffolds are usually two-dimensional (2D) mats having fibers with typically small pore sizes and low thicknesses rather than bulk three-dimensional (3D) scaffolds (Zhao et al. 2020; Si et al. 2014). Therefore, these properties are unfavorable for cell infiltration and complex 3D tissue regeneration (Soliman et al. 2010). For tissue reconstruction with specific shapes (i.e., ear and nose), geometries of a 3D scaffold could directly influence cell distribution and nutrient infiltration. Also controlling individual 3D shapes on cell growth, ECM production, and tissue regeneration is significant. In tissue regeneration, the scaffolds that combine the cells to repair the damaged tissue and restore its functions, play a significant role (Chen et al. 2019). The networks of 3D fibrous

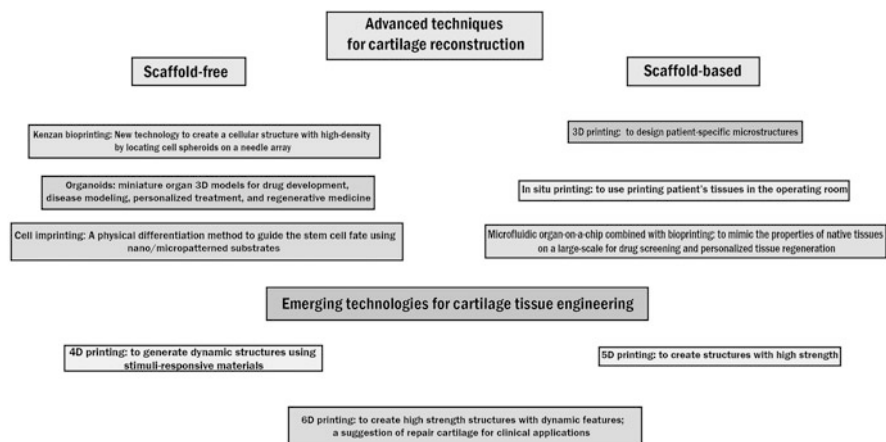


Fig. 9.1 Advanced and emerging technologies and their applications for cartilage tissue engineering

scaffolds should mimic the structure of an ECM and regulate cellular behaviors such as adhesion, differentiation, and matrix deposition (Wei and Ma 2008). The desired response requires a 3D porous structure that support adequate gas exchange, nutrient supply, and growth factors which are essentials in survival of the cells (Farr et al. 2011; Campos et al. 2019). However, the conventional methods are unable to produce precise and customized biomimetic tissues (Jordahl et al. 2018). To tackle these challenges, preparation of 3D porous biomimetic scaffolds in combination with advanced technologies has been suggested in current tissue engineering protocols (Chen et al. 2019). Cartilage structure and its component heterogeneity are crucial characteristics in functional regeneration and repair (Shen et al. 2019). In cartilage tissue regeneration, variety of cell sources and exogenous stimuli have been used that provided an advancement toward replicating the native architecture and functional properties of cartilage tissue (Shen et al. 2019; Daly et al. 2017). This chapter underlines the latest progress in cartilage tissue engineering as well as discussing the advanced scaffold-based and scaffold-free techniques. In Fig. 9.1 some of the advanced and emerging technologies for cartilage regeneration are summarized.

9.2 Scaffold-Based Techniques for Cartilage Regeneration

An ideal scaffold in cartilage tissue engineering should possess similar structural organization to that of native articular cartilage, including sufficient mechanical properties, excellent biocompatibility, and controllable degradation rate (Zhu et al. 2021). Moreover, adjusting cell biological activities such as promoting cell adhesion, proliferation, differentiation, tissue generation, and ultimately repair of

impaired tissues are critical roles of the microenvironment within scaffolds (Cancedda et al. 2003).

The compositions of the scaffold, growth factors, and cells may generally be uniformly distributed and the structure of some scaffolds is non-oriented in current designed scaffolds. The inability to precisely control scaffold internal architecture leads to scaffolds with random pore organization (Daly et al. 2017; Sun et al. 2017). For cartilage tissue engineering, the development of scaffolds with materials that exhibit strong mechanical support, biocompatibility, biodegradability, and osteoinductive properties is essential. The appropriate materials to replace native cartilage tissue at damage sites include metals, ceramics, and polymers (Barradas et al. 2011). However, the non-degradability of metallic compounds, the poor degradability of ceramics, and the low mechanical strength of polymers are critical challenges in cartilage tissue engineering. Therefore, to develop scaffolds with optimum properties, the investigators have focused on using composite materials. Biomaterials are employed to produce scaffolds that act as a temporary matrix for establishing a specific ECM for cartilage regeneration (Balagangadharan et al. 2017; Hinderer et al. 2016). A scaffold with a natural and synthetic structure is defined as artificial support that provides a 3D environment for cartilage engineering (Xavier et al. 2015; Wei et al. 2021). Herein, the advanced techniques for cartilage tissue regeneration are discussed, including 3D bioprinting, in situ printing, organ-on-a-chip, and injectable hydrogel-based drug delivery system.

9.2.1 3D Bioprinting

To design an analog microstructure of a 3D native tissue, delivering living cells with appropriate materials in a defined and organized manner is critical (Pati et al. 2014). In various fields, 3D bioprinting as an additive biomanufacturing technology and an emerging trend in therapeutics for fabrication of artificial tissues and organs has been suggested (Cui et al. 2017). Bioprinting closely controls the scaffold characteristics in terms of shape, distribution, and interconnectivity of internal pores (Roseti et al. 2018).

3D bioprinting technology can create layer-by-layer 3D structures directly from a computer-aided design (CAD) that automatically print the piece and fabricate the patient-tailored structure (Khoda et al. 2011). A customized item based on patient medical images (e.g., computerized tomography, magnetic resonance imaging) or a free-form design per demand is obtained by the CAD file (Shen et al. 2019; Daly et al. 2017; Li et al. 2017; Giannitelli et al. 2014; Murphy and Atala 2014). In order to produce a 3D biological structure, this technology starts with a CAD file for depositing biomaterials and living cells of interest in a predesigned configuration. Afterward, cell-laden constructs would be matured to reinforce and develop the desired tissue lay out (Murphy and Atala 2014; Kačarević et al. 2018; Bandyopadhyay et al. 2018; Satpathy et al. 2018). This technology is mainly used for tissue engineering, disease modeling, and drug-testing applications (Hadisi et al. 2020). The fabricated scaffold by 3D printing is a temporary mechanical shield for

the inner cells to proliferate and produce enough ECM to repair the tissue defect (Xiongfa et al. 2018). 3D printing technology can use the deposition of two or more biomaterials with good biocompatibility and mechanical strength, living cells, and active biomolecules to produce structures with reproducible patterns (Chameettachal et al. 2019a; Prendergast and Burdick 2020).

Ideal printable materials are essential to successfully transfer cells and growth factors to damaged/degenerate tissues, providing temporal mechanical support before the maturation of newly generated tissue (Chameettachal et al. 2019b; Shipley et al. 2009). Extrusion printing, inkjet printing, stereolithography, fused deposition manufacturing, direct femtosecond laser writing lithography, laser-induced forward transfer, or powder-based 3D printing are types of bioprinting technologies for printing a 3D construct. The most popular approaches for cartilage bioprinting are extrusion and inkjet printing due to the cost-effective equipment and flexible adaptation to different working environments (Xiongfa et al. 2018). Inkjet printing is capable of much finer droplets but is usually limited by precision on droplet size due to the microelectromechanical system-based print heads (Chameettachal et al. 2019a; Scoutaris et al. 2016; Bishop et al. 2017). In extrusion printing, pneumatic or mechanical pressure bioinks are extruded to form a continuous filament through a microscale needle for intricate geometries with controlled porous architecture (Zhang et al. 2016; Lipson and Kurman 2013). Due to generating large backpressures and the mechanical nozzle systems, cell viability and functionality are decreased (Ozbolat and Hospodiuk 2016). The structural integrity of extruded bioink is generally better than the other techniques because of the deposition of continuous filaments and physiological cell densities (Murphy and Atala 2014; Chang et al. 2011; Malda et al. 2013). Unlike an inkjet printer, extrusion technology has potential for printing a high density of cells and biocompatible materials such as complex polymers having a wide range of viscosity (Chameettachal et al. 2019a; Scoutaris et al. 2016; Mandrycky et al. 2016). For the successful development of functional organs or tissue constructs, bioinks are considered as a significant component of 3D-bioprinting technology (Groll et al. 2018). Supplying sufficient biological cues and mechanical support for the cells and meeting physical and mechanical demands are significant roles of a bioink to complete the bioprinting process (Gopinathan and Noh 2018). A bioink is defined as a formulation of cells in the form of single cells, coated cells, and cell aggregates (of one or several cell types) that may also contain biologically active components and biomaterials (Groll et al. 2018). Generally, the ideal properties of inks are printability, functionality modifications, mechanical properties, non-toxicity, degradability, adhesivity to cells, and porosity (Gopinathan and Noh 2018; Theus et al. 2020). In recent years, the development of appropriate bioinks has received increasing attention, as such systems are required to fabricate complex and highly customized cell-laden bioprinted scaffolds (Valot et al. 2019).

Cell sources such as bone marrow stem cells, allogeneic stem cells, and chondrocytes are used for cartilage bioprinting. Also, co-culturing various cells to solve the shortage of cell numbers and improve differentiation toward cartilage would be helpful (Xiongfa et al. 2018).

For printing a 3D cartilage analog, a variety of synthetic and natural materials have been used. Natural materials include decellularized extracellular matrix (dECM), hydroxyapatite, silk fibrin, collagen, gelatin, and alginate hydrogels. Synthetic materials include polycaprolactone, polyacrylamide, chitosan, polyurethane, hyaluronic acid methacrylate, and poly(ethylene glycol) dimethacrylate (Kačarević et al. 2018; Xiongfa et al. 2018; He et al. 2020). Although low cytotoxicity and superior biocompatibility of natural materials like hydrogels are preferred, they lack enough strength and fail to resist mechanical stress of encapsulating chondrocytes (Visser et al. 2015; Agrawal et al. 2013; Xu et al. 2012). In addition to various cell sources and biomaterials, biochemical factors such as fibroblast growth factor-2 and transforming growth factor can be used to form a highly mimetic macroscale cartilage (Man et al. 2016). Compared to other tissue engineering strategies, optimizing mechanical properties such as mechanical strength, friction, and elastic modulus for cartilage bioprinting will qualify the 3D printed object for load-bearing applications (Xiongfa et al. 2018). Additionally, combining various natural and artificial materials with different stiffness would have a great impact on the mechanical properties, printability, zonal design, and adjustment of the biological performance of the cells. This would help to build a stable cartilaginous biocompatible structure with viable cells that are able to secrete proper cartilage-specific factors (Xu et al. 2012; Oussedik et al. 2015; Yao et al. 2015; Izadifar et al. 2016; Levingstone et al. 2014).

Table 9.1 summarizes some of the studies on 3D bioprinting technology for cartilage tissue engineering.

9.2.2 In Situ Printing

For many innovative techniques and opening new avenues in regenerative therapeutics, 3D bioprinting is an emerging technology. 3D bioprinting can design and fabricate grafts for in vitro/in vivo studies. However, for implantation of these structures in humans, safety risks and complicated manufacturing equipment are required. Furthermore, maintaining the structural integrity of printed constructs for implantation is very significant. In situ 3D bioprinting is an innovative biofabrication technology to circumvent these issues for directly printing structures at the site of injury or defect. The surgeon could use this method to implant construction at the time and location of need (Di Bella et al. 2018). This approach has great potential to produce custom-made grafts that is fabricated from fresh autologous cells, resemble target tissue, and fit precisely in defects. Moreover, the natural cell microenvironment in the body can be harnessed for tissue maturation resulting in tissue regeneration and repair (Ashammakhi et al. 2019). The examples of in situ printing technologies divided into robotic and handheld modes for cartilage tissue engineering are discussed.

Table 9.1 3D printing techniques for cartilage tissue engineering

Utilized materials for 3D printing	Cell types	In vitro/ in vivo	Outcomes	References
SF-dECM	BMSCs	Nude mice	SF-dECM bioinks had potential to fabricate good internal pore structure constructs with appropriate mechanical strength and degradation rate. Obtained constructs were suitable to support BMSC proliferation and promote chondrogenic differentiation. SF-dECM bioinks released TGF-β3, had potential to promote chondrogenic differentiation of BMSCs, and provided a good cartilage repair environment. This study suggested an ideal scaffold for cartilage tissue engineering	Zhang et al. (2021)
Gelatin/PLGA electrospun fibers	Chondrocytes	Nude mice	3D-printed scaffolds which were constructed by combining the 3D printing and freeze-drying, electrospun fiber-based inks have large pores and precisely controlled shapes. They had good elasticity and water-induced shape memory. This study combined scaffold with chondrocytes for cartilage regeneration and shape maintenance in vivo	Chen et al. (2019)
Chitosan-EDTA with physical crosslinking by calcium solution	Chondrocytes	In vitro	A modified chitosan-based 3D printing bioink was exhibited a favorable mechanical property and promoted cell attachment and chondrogenic gene expression in chondrocytes. This study has offered a bioink for 3D bioprinting in cartilage tissue engineering	He et al. (2020)
Assembly of Alg-Gel microspheres in 3D-printed PCL scaffolds	BMSCs	In vitro	The scaffold assisted to differentiate BMSCs toward a chondrogenic phenotype	Xu et al. (2019)

(continued)

Table 9.1 (continued)

Utilized materials for 3D printing	Cell types	In vitro/ in vivo	Outcomes	References
3D printed depth-dependent gradient PCL impregnated with ALMA	BMSCs	In vitro	A potential scaffold for enhance cartilage tissue engineering was demonstrated	Cao et al. (2021)

SF-dECM silk fibroin and decellularized extracellular matrix; *BMSCs* bone marrow mesenchymal stem cells; *PLGA* poly (lactic-co-glycolic acid); *EDTA* ethylenediaminetetraacetic acid; *Alg-Gel* alginate gelatin; *PCL* poly(ε-caprolactone); *ALMA* methacrylated alginate

9.2.2.1 Handheld

The handheld mode is suitable for constructing simple structures with superficial positions and minor damage (Ma et al. 2020). The unit of bioprinting in this mode is syringe-like and driven by a person. Since the shape of a bioprinting object is wholly based on the motion of the operator’s hand, predefined printing paths and geometry are unnecessary (Di Bella et al. 2018). Sterilization and clinical location by handheld mold are performed easier than in robotic mode. However, to achieve multi-biomaterial 3D bioprinting, its application in repairing complex tissues is limited (Singh et al. 2020). There is a report on development of a handheld 3D printing device (biopen) to repair a chondral defect in sheep (Di Bella et al. 2018). They performed simultaneous coaxial extrusion of bioscaffold and cultured cells directly into the cartilage defect in a single-session surgery. There are evidences that show in vivo bioprinting with cells and scaffolds in a large animal model for regeneration of articular cartilage is possible.

9.2.2.2 Robotic

A three-axis portable system drives the motion of the bioprinting unit in robotic mode. This device can generate a printing path using a 3D bioprinting slicer software. Based on the predefined 3D geometry and complex internal structures, the shape of a printing objective can be accordingly designed. The robotic mode is desirable for the precise reconstruction of tissues (Ma et al. 2020; Mironov et al. 2011). A study reported robotic-assisted in situ 3D bioprinting technology for cartilage regeneration (Ma et al. 2020). A six-degree-of-freedom robot was developed to improve the printing accuracy. Hyaluronic acid methacrylate and acrylate-terminated 4-armed polyethylene glycol were the bioink components. The outcomes of in vitro and in vivo showed that improved robot’s accuracy and the repair of osteochondral defect were occurred. This approach is recommended for improving surgical procedures and promoting cartilage regeneration.

9.2.3 Organ-on-a-Chip Platforms

To study physiological responses to drugs, 2D cell cultures have been used as the common approach (Ahadian et al. 2018). In contrast, microfluidic organs-on-a-chip is a powerful tool with widespread applications, mainly in cell and tissue analysis, drug screening, and disease modeling (Ma et al. 2021; Zhu 2020). Organ-on-a-chip can be integrated with animal models to increase the efficiency and biological relevance of drug screening. Creating organ-on-a-chip models recapitulate the structural, microenvironmental, and physiological functions of human organs (Yu and Choudhury 2019). Chip models have the potential to modulate multiple soluble and physical factors simultaneously over space and time with high precision. This technology could be an ideal platform for (stem) cells to regulate biochemical and biophysical cues and to control stem cell microenvironments (Zhang et al. 2017a). Therefore, the microfluidic devices integrate the biochemical and physical factors to control cell behavior and tissue morphogenesis. Human-derived cells can be used to construct personalized organ-on-a-chip platforms (Ahadian et al. 2018). So far, different organ-on-a-chip platforms from various tissues have been developed. To recapitulate a multifaceted joint tissue microenvironment, joint-on-a-chip (JOC) models are valuable platforms that aid in osteoarthritis research. JOCs create dynamic *in vitro* models to mimic the *in vivo* tissue environment through joint-relevant biomechanical or fluidic integration (Banh et al. 2022). Most current microfluidic platforms are designed and fabricated for particular application and are not suitable for large-scale commercial applications. In addition, the production of large quantities of specific cells is challenging. Therefore, to obtain large tissues and organs within microfluidic channels, developing novel designs and achieving advanced models may be required (Zhang et al. 2017a). Moreover, microfluidic devices have challenge in achieving ECM-like heterogeneous composition of biomaterials (Colosi et al. 2016). Recently, bioprinting technology has been used to fabricate organ-on-a-chip models owing to their ability to print multiple materials and cell types simultaneously. This technology can create a biomimetic microenvironment with heterogeneous 3D structures. As mentioned, it is possible to directly print multiple cell types and biomaterials on a microfluidic chip that would be helpful to study transport of nutrition, gaseous exchange, and removal of waste and creating a biomimetic niche (Yu and Choudhury 2019).

A single-step fabrication of a 3D transparent microfluidic chip using a stereolithography-based desktop 3D printer and an industrial 3D printer is reported (Knowlton et al. 2016). This method was used to print functional 3D microfluidic chips. The capabilities of 3D-printed microfluidic devices could be enhanced by coupling 3D cell encapsulation and spatial patterning within photocrosslinkable gelatin methacryloyl. This platform was applied to fabricate a 3D culture environment for long-term cell culture and growth. 3D-printed microfluidic chips could control 3D cell culture environments and advance the applicability of 3D printing to physiological engineering systems for bioengineering applications (Fig. 9.2).

An attractive study presented an integrated tissue–organ printer (ITOP) to fabricate stable, human-scale tissue constructs in any shape (Kang et al. 2016). Defined

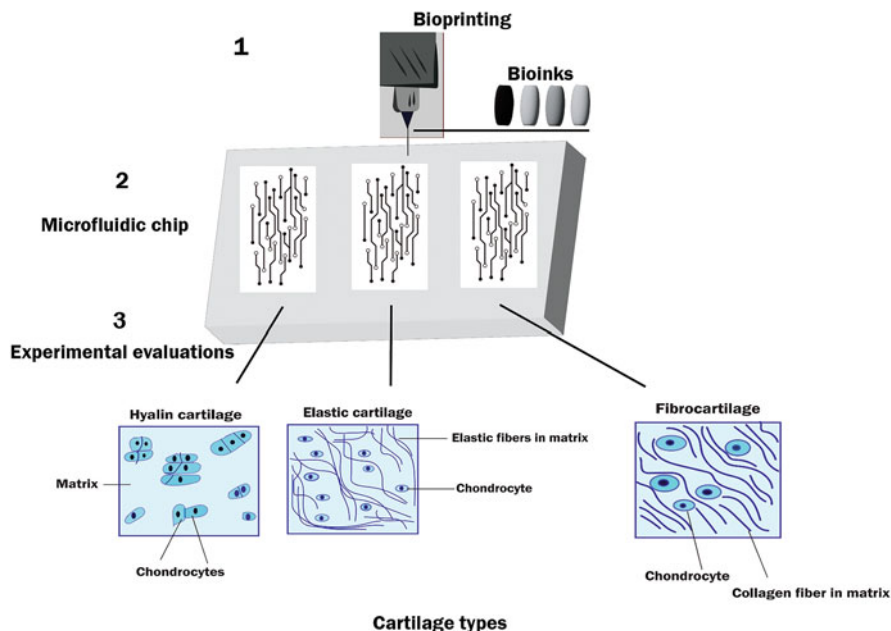


Fig. 9.2 A schematic of the 3D printed cartilage constructs with microchannels. (1, 2) Bioprinter set up using a 3D computer-aided design of human cartilage in the presence of microchannels. (Bioinks can include chondrocytes, growth factors, and biomaterials-like cartilages). (3) The inner environment of cartilage after culturing process and experimental evaluation. (The chondrocytes and cartilaginous matrix in the newly formed tissue are similar to native cartilage in schematic image). This figure was made by a free licensed web: www.vecteezy.com web-site

mechanical stability was obtained by printing cell-laden hydrogels along with biodegradable polymers in integrated patterns. This study evaluated the potential of ITOP to fabricate tissue constructs with complex shapes such as human ear cartilage. Based on the geometry of a computerized tomography image of ear, a motion program was developed to print rabbit ear chondrocytes-laden hydrogel, polycaprolactone, and Pluronic F-127 composite hydrogel. Human ear-shaped cartilage constructs were fabricated with microchannels demonstrating enhanced tissue formation. Native human ear tissue was employed as a positive control. In the newly formed tissues, the cells had similar morphological characteristics to those in native ear cartilage as they located within typical chondrocyte lacunae. Finally, *in vivo* experiments consist of histological analysis, glycosaminoglycan content, vascularization, biomechanical analyses, and resilience measurement were performed. The results showed maintenance of the shape, with substantial cartilage formation and increase of Glucosamine after 2 months. The inner regions were avascular similar to native cartilage and the cartilage cells were viable which shows an adequate nutrient diffusion during development. Collectively, the resilience measurements

demonstrated that the generated ear-shaped cartilage had resilience properties similar to those of native cartilage (rabbit ear). It is concluded that ITOP has potential to create 3D free-form shapes with multiple types of cells and biomaterials to form various tissue types. This technology enables us to produce proper functional tissues and organs of numerous cell types at precise locations for clinical applications.

Sensory systems were also considered to analyze metabolites and other organ-on-a-chip outputs in a high-throughput manner (Ahadian et al. 2018). It is also essential to integrate a sensor with organ-on-a-chip platforms for monitoring the performance of tissues and the extracellular environment (Zhang et al. 2017b). Most of the integrated sensors into organ-on-a-chip devices are comprised of three components, a detector where analytics bind, a transducing part that converts binding events to create signals, and a signal processing device to amplify and convert signals into appropriate reading (Mousavi Shaegh et al. 2016). However, integration of these components remains challenging in sample preparation, biocompatibility, and system integration. Despite the myriad advantages of organ-on-a-chip platforms, there are still many concerns that exist before they can be used systematically as drug screening platforms (Ahadian et al. 2018).

9.2.4 Injectable Hydrogel-Based Drug Delivery System

Chronic degenerative diseases such as osteoarthritis that affects the knee, distal phalangeal, and intervertebral joints can be treated using oral medications such as nonsteroidal anti-inflammatory drugs (NSAID) or corticoids (Barbucci et al. 2002). However, as most used current treatments, these medications can be accompanied by different adverse effects such as gastrointestinal reactions or renal dysfunction (He et al. 2017a). Currently, to resolve cartilage diseases such as severe osteoarthritis, conventional surgery methods like microfracture are being used (Steadman et al. 2010). Microfracture creates a perforate in the subchondral bone, which may be caused bleeding and the formation of a clot rich in growth factors and mesenchymal cells. This technique regenerates fibrocartilage with worst physical properties than the original. Other methods are considered to address some of these impediments to surgery-based strategies for treating cartilage diseases (Gomoll et al. 2010; García Fernández et al. 2020).

Hydrogels, with several advantages, are one of the emergent candidates in cartilage regeneration. They are capable of notable swelling and possess porous materials with 3D polymeric networks (Li et al. 2019a). The hydrogels that process outside the patient were initially implanted using invasive surgical techniques. Although hydrogels have a flexible structure and their rheological performance is excellent, the feasibility of using hydrogels remains limited due to their poor mechanical strength and delicate structure. Injectable hydrogels could provide good biocompatibility, desirable biodegradability, low cytotoxicity, and a highly hydrated 3D structure analogous to cartilage ECM (Liu et al. 2017; Sun et al. 2020). The 3D porous structure of injectable hydrogels easily improves the supply of nutrients and cellular metabolites. The application of injectable hydrogels is a

practical method that can be done in a minimally invasive process via either direct injection or arthroscopy (Li et al. 2019a; Dimatteo et al. 2018). Different examples of natural polymers are hyaluronic acid, collagen, gelatin, alginate, chitosan, chondroitin sulfate, and fibrin. On the other hand, semi-interpenetrating networks (semi-IPN) hydrogel that mimic ECM features have been suggested for cartilage regeneration (Knowlton et al. 2016). A research study reported the synthesis of an injectable and biodegradable drug delivery system consisting of oxidized dextran (Dex-ox), gelatin, and hyaluronic acid, without the use of any chemical initiator (Brigham et al. 2009). Dex-ox formed a hydrogel in the presence of gelatin and the chains of HA created a semi-IPN which can be utilized as an injectable hydrogel. Two anti-inflammatory drugs including naproxen and anti-inflammatory corticosteroid were used to load the hydrogel. These drugs are widely used to inhibit inflammation and cartilage damage in osteoarthritis. The injection of the loaded hydrogels in the knees of New Zealand rabbits' models with osteoarthrosis indicated the effectiveness of injectable hydrogel-based drug delivery system (IHDDS).

Other analyses revealed cartilage preservation in both drug-loaded systems and expression of collagen type II in the ECM. These promising results suggested that the IHDDS can be a great alternative for current treatments of osteoarthritis and should be investigated in further details. Moreover, injectable hydrogels can be used to deliver bioactive molecules and encapsulate cells through stimuli-responsive release mechanisms into targeted sites for cartilage regeneration (Li et al. 2019a).

During the last decades, advanced hydrogels such as hydrogel materials with injectable and self-healing properties as well as hydrogels with higher stability have been the main focus of the research (Tu et al. 2019; Chai et al. 2017). Self-healing materials are smart materials with stimulus-responsive functional groups for automatic damage repair without external interventions (He et al. 2017b). For example, in thermosensitive injectable self-healing hydrogels, the sol-gel transition of thermosensitive hydrogel is initiated by a rise or decrease of temperature. Thermosensitive hydrogels can embed cells in the gel, fill irregular cartilage defects, and prevent undesirable precursor solution diffusion. Their gelation can be easily triggered under a mild physiological conditions without any organic solvents in a harsh environment (Liao et al. 2018). The gelation temperature is regulated by a delicate hydrophilic-hydrophobic equilibrium that may be adjusted by composition and concentration. The thermosensitive hydrogels' sol-gel transition temperature should be controlled between ambient (20 °C) and physiological (37 °C) to meet the needs of biomedical applications (Klouda and Mikos 2008). Various natural macromolecules and synthetic polymers are employed to fabricate hydrogels and prepare a biomimetic microenvironment for cell encapsulation, proliferation, and differentiation (Naahidi et al. 2017; Hoffman 2012). Thermosensitive hydrogels may be created using both natural macromolecules (e.g., collagen, gelatin, and chitosan) and synthetic polymers (e.g., polyesters, polypeptides, polyethers (Pluronic), and poly(N-isopropylacrylamide) (PNIPAAm)). They are suitable for therapeutic applications since they have the potential to considerably reduce patient pain, infection, recovery time, and treatment cost (Bakaic et al. 2015). Advanced hydrogels have long been recognized as a suitable substrate for cartilage

regeneration (Li et al. 2019a). These biomaterials have shown remarkable beneficial effects in wound healing as well as drug delivery systems (Rizzo and Kehr 2021; Vashist et al. 2017). There is a report on the possibility of in vitro 3D cartilage regeneration using moldable thermosensitive hydroxypropyl chitin (HPCH) hydrogel and the in vivo destiny of the resulting cartilage (Xu et al. 2020). Goat auricular chondrocytes were encapsulated in the HPCH hydrogel to create a chondrocyte-hydrogel complex. The results indicated that the HPCH hydrogel had acceptable gelation characteristics (18 s at 37 °C), biocompatibility (cell number nearly doubled after 1 week), and the capacity to be used as an injectable hydrogel. All in vitro-cultured structures retained most of their original forms and exhibited typical cartilaginous characteristics such as numerous lacunae and matrix deposition. With prolonged in vivo implantation, these in vitro samples matured and mostly retained their original forms. These findings showed that the moldable thermosensitive HPCH hydrogel might be a promising substrate for cartilage regeneration both in vitro and in vivo. Therefore, designing various forms of hydrogels with novel properties including a high density of chondrocytes for creating cartilage-like tissue should be highly considered. Some of the studies on advanced injectable hydrogels for cartilage regeneration are summarized in Table 9.2.

Notably, implanting injectable gels near or at the precise target site must be performed with care. Also, sol-gel transition needs to be in a controlled manner. For example, the gelation should not be too slow if the pre-gel is not viscous. On the other hand, a quick sol-gel transition of pre-gel during the injection might lead to the stuck of the fluid in the needle. In addition, high injection force needs to inject a fluid with high viscosity, which will lead to clinicians' hand fatigue and discomfort of patients (Sun et al. 2020). Injectable hydrogels that being implanted in hard tissue such as bone and cartilage are for filling and delivery applications since they do not have enough strength for repair of the damaged tissue. Having ideal mechanical properties especially compressive modulus is crucial in scaffold of cartilage or bone tissue engineering (Liu et al. 2017). Therefore, to administer a hydrogel in the body, an injection system with suitable viscosity, desired gelation kinetics, and defined mechanical properties is required. Developing devices to transport injectable materials at the target site can effectively tackle these challenges. Future researches should focus on the reducing the distance of the pre-gel transport without changing viscoelasticity and to improve the modulus of injectable hydrogels. The development of advanced techniques to inject hydrogels with proper mechanical properties for cartilage regeneration would also be precious (Sun et al. 2020).

9.3 Emerging Technologies for Cartilage Tissue Engineering

To design and fabricate 3D architectures with higher similarity to native tissues, developing new technologies as the next generation of tissue engineering is essential. In this section, the promising approaches, including 4D/5D/6D printing, that might be accompanied by partnered research efforts within academia and industry are suggested for cartilage tissue engineering.

Table 9.2 A summary on the injectable hydrogels for cartilage regeneration

Injectable hydrogel components	Encapsulated agents	In vitro/ in vivo	Biological effects	References
Chemical modification of chitosan using N-BMPS and β -GP	KGN with pro-chondrogenic affect	In vitro	Based on the enhanced hydrogel shear modulus, gelation behavior, long-term drug release, this thermosensitive hydrogel was reported as a promising candidate for cartilage tissue engineering	Dehghan-Baniani et al. (2020)
A thermosensitive chitosan hydrogel which formed on the nanopatterned silk	KGN to promote chondrogenesis of the stem cells	In vitro	An effective nano-engineered system to provide a layer mimicking the superficial zone of articular cartilage. This implantable structure had ability to release drug in a sustained manner for cartilage regeneration	Dehghan-Baniani et al. (2021)
An UV light-reactive, rapidly cross-linkable matrix integrated with drug-loaded nanoparticles	KGN to promote BMSCs into chondrocytes	Rabbit model	Injectable hydrogel scaffold with sustained-release property of KGN was capable to fill of the defects and generate hyaline cartilage close to the native tissue	Shi et al. (2016)
Chitosan-based nanocomposite thermogel with superior shear modulus resembling cartilage	KGN and DS with dual pro-chondrogenic and anti-inflammatory functions respectively	In vitro	The outcomes were including thermogel strength and ability to co-deliver anti-inflammatory and chondro-inductive biomolecules. This system is a great candidate in functional cartilage tissue engineering	Dehghan-Baniani et al. (2022)

(continued)

Table 9.2 (continued)

Injectable hydrogel components	Encapsulated agents	In vitro/ in vivo	Biological effects	References
Thermosensitive hydrogels: methylcellulose, xanthan gum, and/or carboxymethyl chitosan	Three different drugs were incorporated into the hydrogels: dexamethasone, diclofenac sodium, and gallic acid	In vitro	The blends injectable hydrogels could be effective candidates for minimally invasive applications in cartilage tissue engineering and drug delivery systems	Westin et al. (2020)
Thermosensitive hydrogels based on PIPAM/hyaluronic acid containing CH-g-AA coated PLGA-ACH micro/nanoparticle	Melatonin	In vitro	This injectable hydrogel was suggested as a promising material system for cartilage tissue engineering due to the improving mechanical properties, low syneresis, the releasing drug in a sustained manner, and high bioactivity	Atoufi et al. (2019)
PF127/GAG	BMP-2 to mimic ECM	Rat	PF/GAG@BMP-2 proposed as an injectable and thermosensitive hydrogel for clinical recovery of injured cartilage tissue engineering	Li et al. (2018)
Poly(ester-amide) polymer, methoxy poly(ethylene glycol)-poly(pyrrolidone-co-lactide) diblock copolymer	Chondrocytes	In vitro	The results have shown that this thermosensitive hydrogel can be applied as a potential injectable scaffold for cartilage tissue engineering	Fu et al. (2018)
Fabricated system through the thiol-ene reaction between HA-SH and HB-PEG macromer	CPCs with the capability of self-renewal and multilineage differentiation	In vitro	This in situ forming and injectable hydrogel as a CPC delivery carrier had excellent potential by accelerating ECM production and retaining the phenotype and function of encapsulated CPCs.	Li et al. (2020)

(continued)

Table 9.2 (continued)

Injectable hydrogel components	Encapsulated agents	In vitro/ in vivo	Biological effects	References
			This hydrogel system was considered for the treatment of degenerative knee diseases by cartilage regeneration	
CS-SH/HB-PEG injectable hydrogel scaffolds	ADSCs with great cell viability and effective to improve chondrogenesis in CS-SH/HB-PEG	In vitro	The results suggested that the CS-SH/HB-PEG injectable hydrogel had potential to suppress the pro-inflammatory cytokines, and physiology function of encapsulated ADSCs. This could be efficient for cartilage tissue engineering via a simple and minimally invasive procedure	Li et al. (2021)
HBC/OCS injectable hydrogels	ADSCs	Mice	A composite biomimetic hydrogel obtained by 3D bioprinting technique. Having formability and biocomposite characteristics made it a great candidate for cell delivery in cartilage regeneration	Li et al. (2019b)
A nanocomposite a-CNC/collagen hydrogel	MSCs	Nude mice model	Injectable collagen-based nanocomposite hydrogel with self-healing and fast shear thinning properties was used to protect cells during injection, fit into the irregular cartilage defect, and deliver MSCs for cartilage regeneration through minimally invasive procedures	Zhang et al. (2020)

(continued)

Table 9.2 (continued)

Injectable hydrogel components	Encapsulated agents	In vitro/ in vivo	Biological effects	References
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BMPS N-(β -maleimidopropoxy) succinimide ester; *β -GP* β -glycerophosphate; *KGN* kartogenin; *UV* ultraviolet; *BMSCs* bone marrow-derived mesenchymal stem cells; *DS* diclofenac sodium; *PNIPAM* poly(N-isopropylacrylamide); *CH-g-AA* chitosan-graft-acrylic acid; *PLGA* poly(lactic-coglycolic acid); *PF127* pluronic F127; *GAG* glycosaminoglycan; *BMP-2* bone morphogenetic protein; *ECM* extracellular matrix; *CPCs* cartilage-derived progenitor cells; *HA-SH* Thiol-functionalized hyaluronic acid; *HB-PEG* hyperbranched poly(ethylene glycol) multiacrylatemacromer; *CS-SH* thiol-functionalized chondroitin sulfate; *ADSCs* adipose-derived mesenchymal stem cells; *HBC* hydroxybutyl chitosan; *OCS* oxidized chondroitin sulfate; *CNS* cellulose nanocrystals; *MSC* mesenchymal stem cell

9.3.1 4D Printing

In biomedical applications, most 3D-printed biomedical structures cannot adequately mimic the dynamic human tissues. These structures are mainly static and inanimate and lack the time-dependent dimension (Lui et al. 2019; Saska et al. 2021). Designing the printed constructs by developing 4D printing technology has created a revolution where time is incorporated into the conventional concept of 3D printing as the fourth dimension (Miao et al. 2016). 4D bioprinting as an emerging technology for constructing dynamic, personalized, and precise tissue engineering scaffolds is vital (Su et al. 2018). In 2014, the concept of 4D printing was introduced (Tibbits 2014). In this technology, 3D constructs have been fabricated based on three dimensions (*x*, *y*, *z*) by combining living cells, bioactive factors, and programmable functional biomaterials that can change their physical structure over time (Chen et al. 2022). This technology uses smart biomaterials that respond to environmental stimuli (humidity, temperature, chemicals, magnetic and electric field, Osmolarity, and ultraviolet light and transform their morphology or function (Saska et al. 2021). For example, an intelligent temperature-responsive hydrogel, alginate/PNIPAAm was considered for 4D printing due to its high toughness and volume reversibility in response to environmental changes (Bakarich et al. 2015).

This strategy has capability to mimic the dynamic biological behaviors or intricate geometry of native tissues for tissue regeneration with functional maturation properties and the fabrication of medical devices (Wang et al. 2021; Yang et al. 2019). Betsch, et al. have presented an advanced strategy by incorporating 4D into a drop-on-demand 3D bioprinter to remodel collagen-based bioinks in real-time during the bioprinting process (Betsch et al. 2018). They fabricated functional bioinks by aligning collagen fibers. Streptavidin-coated iron nanoparticles were placed in printable bioinks with different concentrations of low gelling temperature agarose and type I collagen. A magnetic-based mechanism was employed during bioprinting of the hydrogels. This mechanism was applied to align collagen fibers in less concentrated hydrogel blends with a maximum agarose concentration of 0.5% (w/v). Hydrogel blends with unidirectionally aligned collagen fibers had

significantly higher compression moduli than printable blends with more enhanced agarose concentrations which have shown random collagen fiber. Bioprinted constructs with alternating aligned and random fiber layers were fabricated for cartilage tissue engineering applications. After 3 weeks of culture, cell-loaded constructs with alternating layers of aligned and random fibers express more collagen II than fiber constructs with random orientation. This study showed the significance of the structure and architecture of bioinks used in bioprinting for tissue engineering and personalized medicine applications. Generally, it is essential to note that precisely controlling the transformation of printed objects using 4D bioprinting at an early stage of development is still difficult.

9.3.2 5D Printing

In 2016, 5D printing was introduced as an emerging technology for printing complex functional structures (Zeijdeveld 2018). In this technology, the objects are printed in five different axes: x , y , and z -axis; two additional axes include rotation of the print bed and rotation of the extruder head (Anas et al. 2022). Instead of flat layers in 3D printing, 5D printing can be generated curved layers with improved strength (Haleem et al. 2019). The print part moves to create a curve layer while the printer head prints. Existing the force of material during a curved layer can be fabricated the more potent printable object. 5D printing can be used a lesser raw material than 3D printing to make implants of the same strength (Reddy and Devi 2018; Gillaspie et al. 2016). Therefore, this technology has great potential for generating complex-shaped implants to fulfill the immediate requirements of medical and orthopedics (Haleem et al. 2019). However, despite the higher applicability of 5D printing in the medical industry, the printed objects in this method could not be responsive to stimuli as in 4D printing (Anas et al. 2022).

9.3.3 6D Printing

In 2021, the new exciting concept of 6D printing was introduced. In 6D printing, the concepts of both 4D and 5D printing techniques are employed. In this method, the object could be printed in five degrees of freedom using innovative and smart material. 6D printed objects are more robust, effective, and uses less material in manufacturing rather than 4D printing materials (Georgantzinos et al. 2021; Ghazal et al. 2022). This novel technology will increase product efficiency, resulting in low material cost and is of interest in cartilage tissue engineering for production of lighter and more substantial parts (Fig. 9.3).

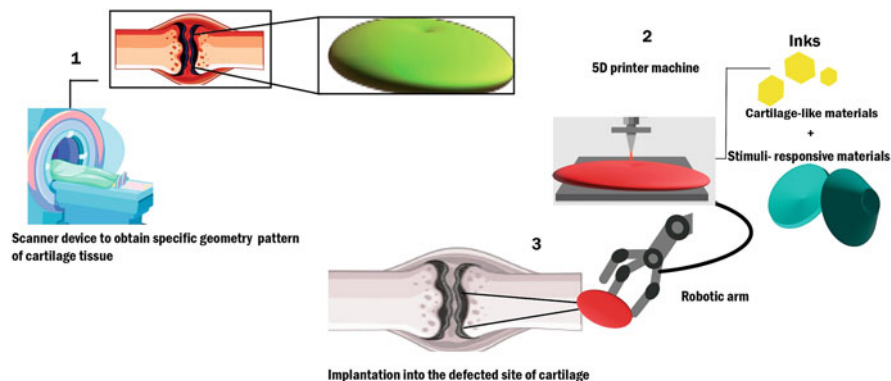


Fig. 9.3 A fabrication process for a customized cartilage implant by 6D printing technology. (1) Obtaining cartilage geometry of patient. (2) Construction of specific-patient implant using a 5D printer equipped with a robotic arm using cartilage-like materials and innovative inks. (3) In situ implantation of the customized implant into the defective cartilage tissue. This figure was made by a free licensed web: www.vecteezy.com web-site

9.4 Scaffold-Free Techniques for Cartilage Regeneration

Most of the cells in the body require ECMs to obtain structural and mechanical support, including rigidity and elasticity (Chan and Leong 2008). Bio-scaffolds with defined features have been used extensively in tissue recovery and mimicking endogenous niches. However, these structures may be accompanied by challenges such as reduced cell survival and proliferation due to limited intake of nutrients and cells inside the scaffold (Chan and Leong 2008; Luo et al. 2018). Moreover, scaffold implantation, toxicity of biomaterials, and impaired host integration are considered the scaffold-based techniques limitations (Grogan et al. 2020). Therefore, alternative methods based on scaffold-free techniques can be employed. According to the definition, “scaffold-less bioprinting refers to the platform without requiring cell-laden or adherence within an exogenous 3D construct.” Scaffold-free techniques are considered as one of the main techniques for the culture of cartilage cells. The cells control ECM production in scaffold-free process to build up the entire construct and mimic the natural growing process (Athanasίου et al. 2013). Generating 3D micro-tissues or spheroids provides conditions for mimicking the native fibrocartilage or articular cartilage. These 3D structures can be employed as modular units for implantation in meniscal and articular cartilage lesions (De Moor et al. 2020). However, maintaining a precise shape of scaffold-free constructs is very important (Grogan et al. 2020). Advanced strategies employed in cartilage engineering include Kenzan bioprinting, organoids, cell-sheet, cell-imprinting, and scaffold-free techniques.

9.4.1 Kenzan Bioprinting

Bioprinting technology has the potential to mimic the 3D microenvironment of the cells and fabricate complex constructs with high resolution and repeatability (Khoshnood and Zamanian 2020). There are, however, limitations in traditional bioprinting methods regarding the use of scaffolding material. For example, selection and use of a suitable material for the bioprinting process that can be supportive for the cell types within a construct and the recipient organism, is challenging. Since each cell type needs to be embedded in a different type of hydrogel, finding a universal material is yet to be established (Kang et al. 2016; Moldovan et al. 2017). In addition, exposing live cells to high shear stress, overheating, and toxic compounds is intrinsically traumatic even generated from initially cell-friendly materials (Moldovan et al. 2017; Hendriks et al. 2015).

This section introduces scaffold-free methods including living cell aggregates with no carrier for fabrication of functional tissue. To fabricate 3D printed constructs, cell aggregations as bioinks in the forms of spheroid and strand are employed. For example, the microneedles-based (“Kenzan”) spheroid is a type of 3D printing technology based on biomaterials’ free methods. To directly and precisely “print” spheroids, they are placed in stainless steel microneedles (“kenzans”) having predesigned contiguous structures with micron-level precision (Agarwal et al. 2021; Moldovan et al. 2017). These structures would be cultivated until the spheroids fuse into cellular aggregates and form their extracellular matrix which results in tough organized structure. This novel technology reduces printing time on spheroid bioprinting (Moldovan et al. 2017; Jeong et al. 2020) (Fig. 9.4). It is reported that fabricating cartilage constructs is feasible for repairing extensive chondral defects scaffold-free Kenzan method by bio-3D printing (Nakamura et al. 2021). The human-induced pluripotent stem cell (iPSC)-derived neural crest cells was used to undergo chondrogenesis through mesenchymal stem cell differentiation. By optimizing fabrication time during chondrogenic induction, unified cartilaginous constructs with functional properties up to 6 cm² could be assembled. Collective data from the construct’s mechanical strength and molecular expression analyses showed that combining novel iPSCs and bio-3D printers using a Kenzan needle array technology may facilitate the rebuilt of articular cartilage defects.

9.4.2 Organoids

Transplantable organoids have emerged as potential materials to replace and regenerate tissues or organs such as cartilage. Miniature organ models, organoids generated in vitro, are complex 3D stem cells such as adult stem cells, embryonic stem cells (ESCs), and iPSCs (Garreta et al. 2021; Kim et al. 2019; Kratochvil et al. 2019). These 3D structures mimic the specific structural and functional characteristics of a native organ. Since organoids are generated by stem cells derived from the patient tissue, they could produce an isogenic tissue having identical genes.

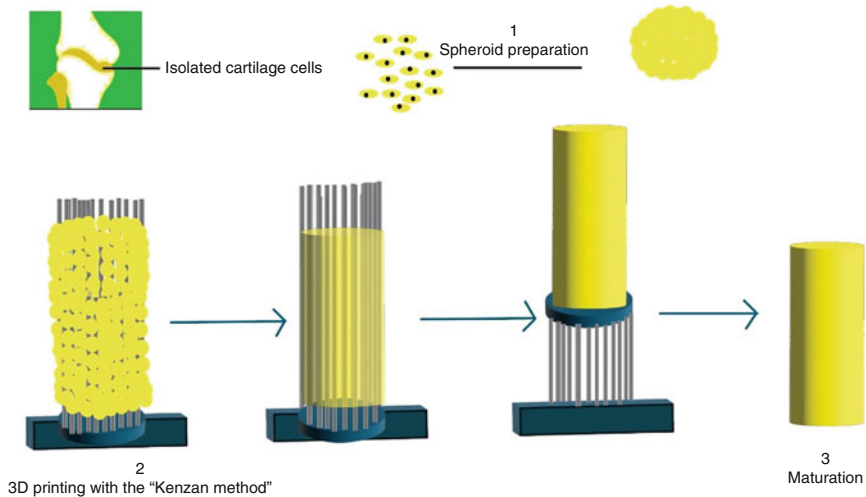


Fig. 9.4 Schematic of “kenzan” bioprinting for cartilage regeneration. (1) spheroid preparation. Cultivating of various cell types correspond to the cartilage tissue. Thousands of these cells are agglutinated to arrange cell spheroid. (2) 3D printing with the “kenzan method.” 3D Stacking of the cell spheroids. (3) Maturation, restructuring, and self-organization for the formation of functional tissue based on the cell types. This figure was made by a free licensed web: www.vecteezy.com web-site

Moreover, organoids could be beneficial for studying the development and disease mechanisms in regenerative therapies (Kim et al. 2023).

With the effort of many researchers in culturing organoids, there are many limitations in current organoid technologies (Kim et al. 2023; Rawal et al. 2021). For example, directing the shape of spheroids and organoids in order to obtain specific size and architecture is difficult considering the standard cell culture methods (Santo et al. 2016). Plus, for clinical application, many organoids having standardized and uniform sizes are required to construct tissues with similar biological properties (O'Connor et al. 2021). However, expanding the culture surface area for mass production of organoids and employing them for high-throughput screening and therapeutic application is not possible. Therefore, expanding the culture surface area for mass production of organoids to be employed for high-throughput screening and therapeutic application is associated with obstacles (Santo et al. 2016). To overcome some of these challenges, 3D bioprinting of organoids has recently emerged (Rawal et al. 2021). The advancement of 3D bioprinting by printing and fusing organoids as bioinks has become possible to construct a tissue in a desired position (Murata et al. 2020). This technology can enhance the complexity and size of organoids and provide tissue-specific geometries and architectures with anatomical and functional similarities to native tissues. The bioprinted tissue organoid structures can be applicable for development and disease modeling, drug screening and discovery, as well as regenerative therapy (Rawal et al. 2021; Bartfeld et al. 2015). Organoid technology can gradually attract the

attention of researchers for drug screening and personalized medicine using autologous stem cells, including adult stem cells, ESCs, and iPSCs. Researchers are interested in customized stem cell organoids to address the transplant immune rejection of artificial organ transplantation. Transplantation of personalized organoids using the fusion of micro/nanoengineering-based maturation, biological factors, and mass production systems provide a great chance of progression (Kim et al. 2023).

9.4.3 Cell Sheet

Cell sheets have attracted the attention of researchers for drug discovery and tissue repair to provide more predictive data for in vivo testing. For constructing 3D tissue-like structures, cultured cells are harvested as intact sheets and their deposited ECM can be transplanted directly into defect site (Alghuwainem et al. 2019). Cell adhesion and detachment processes are performed using a thermo-responsive culture dish to control the hydrophobicity of the surface without the need for any proteolytic enzyme. A non-toxic nanoscale material creates a temperature-responsive culture dish, poly(N-isopropylacrylamide (PIPAAm), to allow cell adhesion and proliferation (Alghuwainem et al. 2019; Elloumi-Hannachi et al. 2010) due to the hydrophobic sheet surface and the dehydrated PIPAAm in temperatures higher than 32 °C. However, when the surface temperature is lower than 32 °C, the cell sheet would detach by hydrating the PIPAAm and making the sheet hydrophilic. Cell adhesion and detachment are reversible processes (Matsuda et al. 2007). When the temperature is below 20 °C, a complete detachment of the cells from the substrate occurs during the cell morphological changes due to the absence of the mediated force between the cytoskeleton and the ECM. The biological functions and structure of cells with ECM integration can be recovered for re-attachment. The bio-functional cell sheet can transplant into host tissues without a scaffold (Alghuwainem et al. 2019; Matsuda et al. 2007; Lu et al. 2019). In the last two decades, the sheets have been employed to regenerate many types of tissues including cartilage (Ding et al. 2021). To make the cartilage cell sheets, commercial temperature-responsive culture dishes are commonly used for the culture of stem cells, chondrocytes, and synovial cells. Continuous cell sheets will form after several days or weeks of culture in temperature-responsive dishes. Harvesting cell sheets is performed by PVDF membranes or forceps upon exposure to 20 °C or room temperature (Lu et al. 2019; Ito et al. 2012; Yano et al. 2013). While the cell-sheet method has becoming a promising strategy for the repair and regeneration of cartilage defects, there are many obstacle and challenges. Maintaining sufficient nutrient and oxygen supply as well as efficient exchange of cellular metabolites is a challenge for the survival of cells in the cell sheets. It is also reported that the components of cells and ECM in the cell sheets could remain different from native cartilage (Kang et al. 2014; Wang et al. 2017). Therefore, fabrication of a cell sheet with a multilayer structure similar to the native cartilage and defined mechanical properties using temperature-responsive is complicated. Various alternative systems have been considered for constructing cell

sheets to tackle these challenges (Lu et al. 2019). For example, it seems that magnetic technology has the potential to fabricate multilayered cell sheets. In this system, multilayered cell sheets can be prepared by magnetically attracting magnetic nanoparticles (MNPs)-labeled cells in a single step. In a magnetic system using different numbers of magnets or magnet patterns, the shape of cell sheets can be precisely controlled (Zhang et al. 2017c). However, the preparation of more complex tissues using existing cell-sheet techniques is still challenging. A novel method named a micropatterned collagen sheet has shown a remarkable outcomes for complex 3D tissue reconstruction (Son et al. 2018). A cell–collagen mixture was constructed and the components of this sheet are more similar to native cartilage using the permeation of a piece of doughnut-shaped paper. However, further research is needed to apply such sheets in cartilage regeneration.

9.4.4 Cell Imprinting

It has been reported that a reliable method for controlling the of stem cells fate could be involved of using nano/micropatterned substrates which is obtained from the templates of the desired cell morphology. In this technique, substrates with imprinted cell-like topographies are transferred to polydimethylsiloxane (PDMS) substrates by mold casting (Moosazadeh Moghaddam et al. 2019). Substrates would resemble the specific topography of the cells' cellular plasma membranes which can be used as a template and consequently dictate the surface of the cells (Mashinchian et al. 2014) (Fig. 9.5). The potency of substrates with imprinted cell-like topographies for direct differentiation of adipose-derived mesenchymal stem cells into chondrocytes has been reported (Mahmoudi et al. 2013). In this study, rabbit adipose-derived mesenchymal stem cells seeded on these cell-imprinted substrates were driven to mimic the specific shape (as determined in cell morphology). Collectively data from molecular characteristics also suggest that presented the imprinted topography of the templates plays a role in the differentiation of the stem cells. The efficiency of this method is 100% in the induction of differentiation if a stem cell that falls into a pattern of a target cell (Moosazadeh Moghaddam et al. 2019). A novel study has reported the efficiency of the imprinting method increased by using microfluidic chip design and application (Kashani et al. 2021). In this

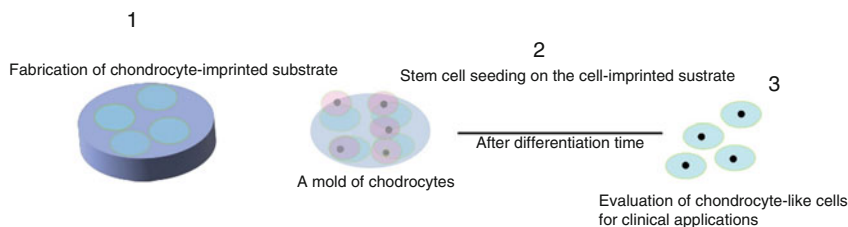


Fig. 9.5 Mimicking chondrocyte-specific topographies by cell-imprinting method to support cell differentiation

method, chondrocytes-imprinted substrates were fabricated using a microfluidic chip. To determine the location of the cells, a microfluidic chip and photolithography were used to fabrication the regular cell culture and to fix them into microchannels. Therefore, the cells were fixed in a specific location for fabrication of the pattern. Soft lithography was used to transform the pattern to PDMS. Stem cells were then seeded into the cell-imprinted substrate using a second microfluidic chip aligned with the substrate. This platform was efficient to precisely place stem cells into the chondrocyte patterns. After 14 days of culture in the chip, the chondrocyte patterns were changed the fibroblast-like morphology of stem cells to the chondrocyte's spherical morphology without using any chemical growth factor. After real-time PCR and immunocytotoxicity analyses, differentiated stem cells were transferred on a collagen-hyaluronic acid scaffold and transplanted into the articular cartilage defect of a 6 months' rabbit. According to the post-transplantation analysis, the articular cartilage defect had been successfully regenerated in differentiated stem cell groups compared to the controls. This study showed the potency of the imprinting method for inducing chondrogenicity in stem cells having the required safety in order to be suggested and considered for clinical trials. This novel method has also been suggested for various cell-based applications including tissue engineering, regenerative medicine, and cell therapy.

9.5 Conclusion

Cartilage is a highly organized, resilient connective tissue. The lack of a blood supply and the low density of chondrocytes makes the self-repair of damaged cartilage tissue a difficult process. Despite many efforts employed to reconstruct adult cartilage tissue, its restoration is associated with challenges. Tissue engineering has great potential for the development of new regenerative therapies. Essential features such as mechanical, electrical, biochemical, and spatiotemporal for generating whole organs and tissues are required. Advanced technologies have been developed to create constructs with functional properties. This chapter has focused on the developmental approaches of scaffold-based and scaffold-free techniques for the fabrication of cartilage tissue. 3D bioprinting to construct tissue structures on demand for transplantation and other biomedical applications is a great achievement. It enables the production of scaffolds with could be precisely placed the cells, biomaterials, and biomolecules into spatially predefined locations. In situ printing is suggested to overcome the possible challenges of 3D printing, including contamination during post-fabrication, and to avoid alterations to the morphology and mechanical integrity of the scaffolds. These could be a great assistance for computer-assisted medical interventions and the development of medical bio-robotics. Moreover, this technology has a remarkable potential for tissue fabrication through natural body's environment which could eliminate the need of bioreactor for tissue maturation. However, it should be remembering that the 3D printed microscale structures could be substantially different from actual tissues and organs. Organ-on-a-chip is another promising development in tissue engineering

techniques that mimic dynamic physiological environment by directing the fluids containing biomolecules, cells, organisms, or chemical agents into a chip. Fabricated structures mainly for drug screening and disease modeling applications are evaluable. The employment of injectable hydrogels as drug and cell delivery systems with minimally invasive procedure is suggested for cartilage regeneration.

Scaffold-free techniques including kenzan bioprinting, organoids, cell-sheet, and cell-imprinting methods for cartilage tissue engineering are discussed. Kenzan bioprinting is a great technology to create cartilage constructs with high cell density along with their ECM. Organoids are attractive models for replicating the complex organ-like physiological microenvironment for personalized medicine and drug screening research. Using biological models along with tissue engineering technology, there is a potential to simulate the complex biological function of the organs in vivo. The cell sheet is considered in regenerative medicine applications due to its scaffold-free nature and its practical preservation of cell-cell connections with their ECM. The cell-imprinting method is considered an efficient way for tissue regeneration by mimicking micromechanical forces or micro/nanotopographical environments to direct differentiation of stem cells into target cells. Also, a cell-imprinted substrate using a microfluidic chip that derived from the cell membrane pattern offers a novel platform to control stem cell fate in various tissue engineering and regenerative medicine applications.

Scaffold-based and scaffold-free techniques have demonstrated satisfactory results for cartilage regeneration. These advanced techniques depending on the defect in cartilage tissue can generate a partial or full-size fragment. Although these techniques hold a great potential for cartilage tissue regeneration, there is an urge in these fabrication techniques to progress with the capability to develop engineered tissues more comparable to native cartilage. For example, fabricating heterogeneous structures of cartilage tissue with defined mechanical properties remains a challenge. Emerging technologies, including 5D printing, are suggested to generate cartilage implants with more mechanical strength.

Moreover, for constructing natural-like cartilage tissue, incorporation of 4D, 5D and 6D printing has been suggested as an excellent strategy. While tissue engineering progress in exponential leaps by introducing advanced technologies, every so often does not fulfill the expected promise of the field. Since the experimental discoveries are always not confirmed by initial clinical trials, emerging technologies to produce end-use products in association with clinical and basic research is an essential requisite.

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Advances in Hydrogels for Cartilage Regeneration

10

Payam Baei, Amirreza Ahmadiasl, Mahsa Ghasemzad,
Samaneh Hosseini, and Mohamadreza Baghaban Eslaminejad

Abstract

The main objective of cartilage tissue engineering is to introduce convenient engineered artificial matrices that can substitute the damaged areas and improve tissue repair. Hydrogels are recognized as promising biomaterials for cell therapy and tissue regeneration applications. Hydrogels with similar structure to native cartilage extracellular matrix (ECM) and possessing proper characteristics such as elasticity, high water content, and bioactivity can be served as convenient

Payam Baei, Amirreza Ahmadiasl, and Mahsa Ghasemzad contributed equally to this work.

P. Baei

Department of Cell Engineering, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

A. Ahmadiasl

Department of Cell Engineering, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Department of Biomedical Engineering, Faculty of Chemical Engineering, Tarbiat Modares University, Tehran, Iran

M. Ghasemzad

Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Department of Molecular Cell Biology-Genetics, Faculty of Basic Sciences and Advanced Technologies in biology, University of Science and Culture, Tehran, Iran

S. Hosseini (✉) · M. Baghaban Eslaminejad (✉)

Department of Cell Engineering, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

e-mail: hosseini.samaneh@royaninstitute.org; eslami@royaninstitute.org

vehicles for implantation of therapeutic cells and drugs into articular cartilage defects in a minimally invasive manner. Hydrogels can be formed with various cross-linking approaches and their physicochemical properties can be tuned by appropriate selection of materials and chemistry. This chapter reviews most recent advances in the development of hydrogels for cartilage tissue engineering by focus on various cross-linking strategies. Moreover, we discuss the methods for designing proper carriers loaded by chondroinductive factors and development of printed bioinks for cartilage regeneration.

Keyword

Hydrogels · Cartilage tissue engineering · Cross-linking · Drug delivery · Bioprinting

10.1 Introduction

Articular cartilage is an aneural and avascular tissue with confined self-regeneration because of slow proliferation of chondrocytes, only specialized cell type found in this tissue. Thereby, several disorders including physical injuries or degenerative diseases such as osteoarthritis may interrupt the normal function of cartilage. To date, various therapeutic approaches such as implantation of stem cells, allografts, and autografts have been introduced for desirable reconstruction of defective cartilage, but each of these techniques confront with specific limitations (Li et al. 2017). Tissue engineering offers optimal conditions for cartilage regeneration by incorporating convenient engineered constructs along with cellular and inductive molecules for resembling the extracellular milieu of native cartilage. Among different types of scaffold for articular reconstruction, hydrogels have attracted special attention (Eslahi et al. 2016; Liu et al. 2017).

Hydrogels with superior viscoelastic features can absorb high amounts of water and represent similar physicochemical properties to natural cartilage ECM. They may be recognized as minimally invasive delivery systems for cells and biomolecules (Yang et al. 2017). Controlled delivery of chondroinductive agents such as small molecules and growth factors can significantly promote the chondrogenesis and cartilage repair. Designing minimally invasive delivery systems for prolonged release of inductive factors to encapsulate cells or damaged regions may result in development of functional constructs (Patel et al. 2019). Advancements in novel technologies such as bioprinting can assist in development of functional three-dimensional (3D) constructs for reconstruction of intricate heterogeneous structures like osteochondral tissue. In this way, designing appropriate hydrogel-based bioinks with desirable biophysical and biochemical features is noteworthy (Radhakrishnan et al. 2017). In this chapter, we focus on the development of hydrogel-based constructs for cartilage regeneration by emphasis

on cross-linking strategies for hydrogel preparation. Moreover, we review the recent advances in hydrogel-based scaffolds as printed bioinks and vehicles for delivery of chondroinductive factors to improve chondrogenesis.

10.2 Cross-Linking Strategies for Hydrogel Formation in Cartilage Tissue Engineering

In cartilage tissue engineering, designing appropriate constructs with desirable biocompatibility and mechanical properties proportional to native tissue is noteworthy. Among various types of scaffolds, hydrogels can be recognized as promising constructs for cartilage reconstruction due to high water content, viscoelastic nature, and structural similarity to cartilage ECM. To date, different types of hydrogels based on various biomaterials have been introduced for articular cartilage repair. Hydrogels can be classified into physical and chemical networks based on the cross-linking mechanism. Physically cross-linked networks form from molecular entanglements or physical interactions, whereas chemically cross-linked networks possess permanent covalent bonds. More details about different cross-linking approaches for cartilage-specific hydrogel preparation are clarified below.

10.2.1 Physically Cross-Linked Hydrogels

Physically cross-linked hydrogels can be formed by typical physical interactions such as electrostatic and hydrogen bonding or by changing external environmental conditions such as pH and temperature. These types of hydrogels may better simulate the physicochemical properties of native cartilage ECM which their constituents are mostly interact with each other by physical bonding. Moreover, owing to the mild gelation conditions and avoidance of toxic chemical cross-linking agent, physically cross-linked hydrogels have been considering special attention. In a study by Park et al., an ionically cross-linkable hydrogel was constructed by conjugating alginate to the hyaluronate backbone via carbodiimide chemistry. Hydrogel formation was occurred in the presence of calcium ions without incorporation of any chemical cross-linking reagent. In order to promote cellular interactions, arginine-glycine-aspartic acid (RGD) peptides were grafted to alginate backbone. By tuning the polymer composition and calcium concentration, the mechanical properties of prepared hydrogel could be regulated. The results indicated appropriate chondrogenesis of chondrocyte-laden hyaluronate/alginate hydrogel following subcutaneous implantation into the dorsal region in a mouse model (Park et al. 2014). Similarly, a hyaluronate-alginate hybrid hydrogel was prepared by grafting alginate with hyaluronate backbone utilizing various types of linkers such as diamines and dihydrazides. Hybrid hydrogel was prepared by physically cross-linking in the presence of calcium ions. Moreover, RGD and histidine-alanine-valine (HAV) peptides were grafted to alginate backbone in order to improve cell-cell and cell-matrix interactions. By increasing the length of linker, the mechanical

stiffness of hydrogel was significantly promoted due to improvement in intermolecular reactions between polymeric chains. Changing the type of linker and consequently tuning the mechanical properties of hydrogel could significantly influence the chondrogenic differentiation of ATDC5, a mouse chondrogenic cell line that was encapsulated within hybrid hydrogel (Seo et al. 2019).

In order to develop a cell-laden polyelectrolyte hydrogel for cartilage regeneration, oligo (poly (ethylene glycol) fumarate) (OPF)-based hydrogel was incorporated with poly(L-lysine) (PLL) polypeptide as an inductive factor for stimulating the chondrogenesis of encapsulated MSCs. The electrostatic interactions between cationic PLL and partial negative charges (i.e., carbonyl groups) on the OPF polymer backbone resulted in a tight interpenetrating network formation and high retention of PLL within OPF hydrogel. The incorporation of PLL polypeptides into synthetic OPF hydrogel, in addition to influencing hydrogel swelling and degradation properties resulted in promotion of type II collagen, aggrecan, and N-cadherin gene expression in encapsulated MSCs (Lam et al. 2016). In an attempt to develop an injectable and rapid self-integrating hydrogel for cartilage regeneration, a shear-thinning hydrogel was constructed based on ureido-pyrimidinone conjugated dextran (DEX-UPy). Its supramolecular hydrogen bonding enabled it to possess shear-thinning and self-healing properties, since these dynamic interactions can deform under stress and reform after eliminating the stress. This self-integrating hydrogel exhibited appropriate injectability and could successfully regenerate the cartilage-bone tissue complex in a subcutaneous implantation model in nude mice (Hou et al. 2015).

Host-guest complexation is a physical interaction which can form supramolecular hydrogels with highly deformable and resilient structure due to the reversible nature. β -Cyclodextrin is a well-known biomolecule acting as host in host-guest interactions. A gelatin-based supramolecular hydrogel was developed by the host-guest interaction between aromatic residues of gelatin (e.g., phenylalanine, tyrosine, and tryptophan) and oligomerized acrylated β -cyclodextrin (Ac- β -CDs).

The hydrophobic cavity of the β -CDs in the hydrogel resulted in efficient loading as well as subsequent sustained release of the hydrophobic drug kartogenin (KGN) and consequently could improve the chondrogenesis of the encapsulated human bone marrow-derived MSCs. The injectable stem cell-laden gelatin supramolecular hydrogels could promote in situ osteochondral regeneration through prolonged co-delivery of hydrophilic and hydrophobic chondrogenic agents. Moreover, implantation of MSC-laden hydrogel in the rat knee cartilage defect model resulted in neocartilage formation (Xu et al. 2019). Similar study represented the formation of host-guest macromers by molecular self-assembly between adamantane-functionalized hyaluronic acid guest polymers and monoacrylated β -cyclodextrins host monomers. Freestanding hydrogel with self-healing capability could efficiently afford the protection of encapsulated stem cells during the compression remodeling as well as rapid adaptation and well-integration with surrounding tissues of the targeted defects. The supramolecular hydrogels could support chondrogenesis of the hMSCs and promote cartilage regeneration in a rat model due to the sustained exposure of encapsulated transforming growth factor beta1 (TGF- β 1) (Wei et al. 2016).

10.2.2 Chemically Cross-Linked Hydrogels

Despite the various desirable features of physically cross-linked hydrogels, their weak mechanical properties limited their application in load-bearing cartilage tissue. Therefore, there has been growing attention in chemically cross-linked hydrogels with superior stability and mechanical properties. In an attempt to develop an injectable in situ forming hydrogel containing two drugs, melatonin and methylprednisolone for cartilage regeneration, chemically cross-linked hydrogel based on alginate and chitosan was constructed. The hydrogel was formed through Schiff base reaction between amine groups of carboxymethyl chitosan and aldehyde groups of oxidized alginate. The chitosan microparticles containing covalently-attached melatonin and physically-dispersed methylprednisolone were incorporated within hydrogel. The hydrogel containing drug-loaded microparticles represented appropriate injectability and sustained drug release for both drugs. This construct revealed desirable chondrogenesis in a rabbit articular defect model (Naghizadeh et al. 2021). A chemical hydrogel composed of glycolchitosan/dibenzaldehyde-terminated poly(ethylene glycol) (GCS/DF-PEG) was developed in order to transplant adipose-derived MSCs for cartilage regeneration. The results of gross and histological scores following 2 months implantation in an articular defect revealed appropriate cartilage repair (Yang et al. 2021). In another effort to prepare a chemically cross-linked hydrogel via Schiff base formation, partially oxidized hyaluronate was combined with glycol chitosan. The degree of oxidation, the mixing ratio between two components, and total polymer concentration were comprehensively influence the viscoelastic properties of prepared hydrogel. Hydrolysis of imine bonds in physiological conditions resulted in hydrogel degradation over 6 weeks. The injectable hydrogel exhibited convenient cytocompatibility in contact with ATDC5 chondrogenic cell line and could be a promising vehicle for chondrocyte encapsulation (Park et al. 2017).

An injectable adhesive hyaluronate-based hydrogel was developed by modifying the polysaccharide backbone with aldehyde and methacrylate groups. The prepared hydrogel exhibited desirable stability and durability within a humid environment over 7 days, along with high adhesive strength. The in situ forming hydrogel could improve proliferation and migration of bone marrow-derived MSCs. In a rat osteochondral defect model, implanted hydrogel exhibited well-integration with adjacent host tissue and significantly promoted cartilage repair during 12 weeks (Chen et al. 2021). In order to evaluate the effect of biochemical and mechanical properties of the material platform on cell interactions and cartilage-specific matrix formation by cells, 39 combinatorial hydrogels based on methacrylated ECM components such as chondroitin sulfate and heparan sulfate were constructed for co-culture of adipose-derived MSCs and neonatal chondrocytes. The mechanical stiffness of hydrogels was adjusted by incorporation of poly(ethylene glycol) dimethacrylate (PEGDMA) and thereby soft (15 kPa), moderate (40 kPa), and stiff (100 kPa) matrices were achieved. The biochemical and histological evaluations demonstrated that the type of ECM component exhibited a significant role in tuning cartilage formation, whereas the stiffness and concentration of ECM molecules

showed lower effects. Among chemical hydrogels based on chondroitin sulfate, soft hydrogels accumulated higher degree of sulfated glycosaminoglycan which could increase the compressive modulus of hydrogels (Wang et al. 2016). In an attempt to design a cartilage ECM-derived hydrogel with mechanical properties proportional to native articular tissue, methacrylation of solubilized decellularized cartilage was performed. The methacrylated cartilage ECM hydrogel represented similar mechanical properties to native porcine cartilage and promoted the growth, ECM production, and significant upregulation of chondrogenic genes in rat bone marrow-derived MSCs following 6-week encapsulation. These results were significantly superior to cells encapsulated within methacrylated gelatin (Beck et al. 2016).

10.2.3 Double-Network Hydrogels

As discussed, despite their various convenient characteristics, physically cross-linked hydrogels possess weak stability and mechanical features with low resistance to loading. Moreover, chemically cross-linked hydrogels are commonly formed via cytotoxic chemical cross-linking agents or polymerization methods which may be detrimental to encapsulated cells. According to these limitations, double-network (DN) hydrogels have been developed by combination of a tight rigid network and a loose soft network which can possess outstanding mechanical properties in comparison with the individual components of the network. The rigid network mainly possesses a covalent cross-linking which can provide stability and shape recovery of construct, whereas the soft network with a physical reversible cross-linking can dissipate the mechanical energy from the applied forces. Thereby, DN hydrogels exhibit remarkable strength, toughness, self-healing, and recoverability which make them promising candidates for cartilage regeneration (Haque et al. 2012).

A tough DN hydrogels were developed by using a two-step photopolymerization procedure including neutral oligo (2,2-dimethyltrimethylene carbonate)-poly(ethylene glycol)-oligo (2,2-dimethyltrimethylene carbonate)-diacrylate (DPD-DA) chains as first ductile network and methacrylated hyaluronic acid chains as brittle second network. The DN hydrogel represented desirable cytocompatibility in contact with swine cartilage chondrocytes. Moreover, great deposition of GAG and collagen type II was occurred over long-term culture as demonstrated by histochemical and immunofluorescence analysis (Fan et al. 2013). In order to achieve a DN hydrogel based on sulfated alginate (SAlg) and chitosan (CS) with high affinity to chondroinductive factors (TGF- β 1 and KGN), the methacrylate and catechol functional groups were conjugated to polysaccharide backbones. In the DN hydrogel, covalent cross-linking of methacrylated SAlg and CS resulted in formation of elastic network, whereas the presence of catechol and amine groups yielded physical and reversible covalent cross-linking and formed sacrificial bonds with energy dissipation. The DN hydrogel with initial compressive modulus of 34.86 kPa are presented desirable compressive strength (421.45 kPa), toughness (89.69 kJ/m³), self-healing, and recoverability. The equal incorporation of binding sites into each of polysaccharide chains resulted in homogenous distribution of cross-linking points

through the network. The great recoverability and self-healing of DN hydrogel attributed to the dynamic and reversible bonds derived from the catechol and amine group. The prepared hydrogel could improve chondrogenesis of encapsulated MSC and chondrocyte co-culture in vitro and appropriately regenerate a full-thickness articular defect in rabbits following 12 weeks (Fig. 10.1) (Baei et al. 2021).

In an effort to develop a functional construct for growth factor-free cartilage regeneration, a polydopamine-chondroitin sulfate-polyacrylamide (PDA-CS-PAM) hydrogel with acceptable tissue adhesiveness and mechanical features was fabricated. The PDA-CS complex was created by self-assembly of PDA and CS due to the numerous reactive catechol groups of PDA, and subsequently, this complex was homogenously inserted into elastic PAM network. The presence of catechol group in hydrogel resulted in great cell affinity and tissue adhesiveness which could assist in cell adhesion and tissue integration. The resulted PDA-CS-PAM hydrogel represented superior resilience and toughness which was desirable for mechanical requirement of cartilage regeneration. The PDA-CS-PAM hydrogel with excellent cell affinity and tissue adhesiveness introduced a growth-factor-free construct, which could support cell attachment, proliferation, and spreading, as well as preservation the phenotype of chondrocytes and inducing the chondrogenic differentiation of bone marrow-derived MSCs. The PDA-CS-PAM hydrogel exhibited an appropriate ECM-mimicking microenvironment to induce cartilage regeneration in a full-thickness articular cartilage defect model (Han et al. 2018). Another DN hydrogel was formed by combination of dynamic covalent chemistry and thermoresponsive engineered proteins. Dynamic covalent hydrazone bond was achieved by mixing hydrazine-modified elastin-like protein (ELP) and aldehyde-modified hyaluronic acid. The formation of the secondary network was occurred by thermal phase transition of ELP. This DN hydrogel exhibited significant slower erosion rate in comparison with the single network hydrogel without thermal cross-linking. The shear-thinning hydrogel protected hMSCs from the mechanically detrimental stress during syringe needle injection and exhibited rapid recovery post-injection. The encapsulated hMSCs distributed homogenously within hydrogel and could retain their capability to chondrogenic differentiation (Wang et al. 2017). A supramolecular DN hydrogel was developed by combination of self-assembled EFK peptides as sacrificial network and covalent polymer networks comprising acrylamide, acrylate-terminated multi-armed PEG and methacrylated hyaluronic acid. The prepared hydrogel illustrated excellent mechanical properties with Young's modulus of 209 kPa and the toughness of 47 kJm^{-3} along with fast recovery. This biomimetic hydrogel resembled the mechanical properties and microstructure of native cartilage. In a full-thickness cartilage defect model in rabbit, DN hydrogel could significantly repair the damaged tissue during 12 weeks (Li et al. 2020a).

In brief, various strategies can be employed for designing hydrogels with desirable features for cartilage regeneration. By considering the limitations of physically and chemically cross-linked hydrogels, DN hydrogels with remarkable versatility can provide the biomimetic features of physical hydrogels as well as mechanical requirements of load-bearing cartilage tissue and compensate the brittleness of chemical hydrogels. The exceptional features of DN hydrogels make them as promising substitutes for cartilage regeneration.

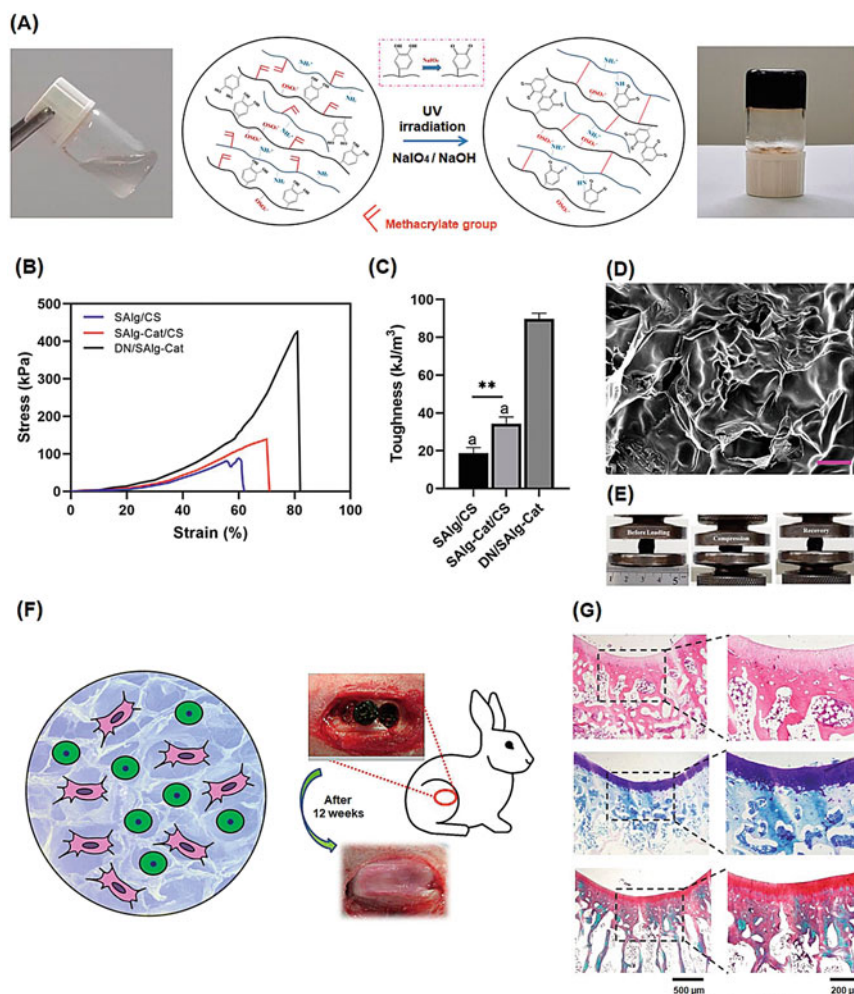


Fig. 10.1 DN Hydrogel Based on Sulfated Alginate (SAIg) and Chitosan (CS) for Cartilage Regeneration. (a) Schematic illustration of the chemical double-network hydrogel (DN/SAIg-Cat). (b) Compressive stress-strain curve of the hydrogels. (c) Toughness of hydrogels obtained by compression testing. (d) Representative SEM micrographs of the cross-sections of the DN/SAIg-Cat hydrogel (Scale bar: 200 μm). (e) Photographs of recovery of DN/SAIg-Cat hydrogel under cyclic compression. (f) Macroscopic appearance of the rabbit knee joint 12 weeks after implantation of the DN/SAIg-Cat hydrogel. (g) Histological examinations of the repaired tissues according to hematoxylin and eosin (H & E), toluidine blue (TB), and safranin O/fast green (SO/FG) staining at 12 weeks post-surgery (Baei et al. 2021)

10.3 Hydrogels as Vehicles for Delivery of Chondroinductive Factors

Application of intra-articular (IA) drug delivery systems could be a promising treatment paradigm compared to the current therapeutic approaches. IA drug delivery systems convey medicinal components to the specified parts via surface modification of drug carriers through sustained-released manner and circumvent off-target effects. Additionally, local bioavailability, stability, efficiency, and half-life of drugs could be accelerated in this method. The IA delivery system can affect the rapid clearance of therapeutic agents from the synovial fluid of the joints due to the thick ECM barrier, so that good drug penetration will occur. In addition, the risk of infection with regular injections can be reduced (Li et al. 2021a; Rabiei et al. 2021). Hydrogel scaffolds with high water content which are made of various natural polymers like fibrin, alginate, chitosan, and hyaluronic acid can be used in IA delivery systems (Rey-Rico et al. 2016). The attractive characteristics of hydrogels, such as appropriate biocompatibility, injectability, self-healing capacity, good elasticity, and degradability, cause them to be used as suitable vehicles for delivery of chondroinductive agents in IA delivery approach (Han et al. 2021). Furthermore, due to high permeability to oxygen and nutrients, hydrogels could provide 3D microenvironment for cell differentiation and open up full remission of cartilage defect (Fig. 10.2) (Ziadlou et al. 2021).

Recently, researches are focused on the use of injectable cell-free hydrogels which can load various types of drugs and bioactive molecules such as kartogenin (KGN), transforming growth factor-beta1 (TGF- β 1), and insulin-like growth factor-1 (IGF-1). These therapeutic agents could promote chondrogenic differentiation and proliferation of MSCs and chondrocytes. KGN is a potential chondroinductive factor which stimulates expression of run-related transcription factor-1 (RUNX1) and reduces its degradation; thereby, it can improve the chondrocyte proliferation. TGF family mainly TGF- β 1 is the other effective cytokine for cartilage repair. It could increase ECM formation, differentiation of chondrogenic cells and reduce OA symptoms. Another popular growth factor is IGF-1 which inhibits ECM degradation, enhances expression of chondrogenic markers, and regulates cell apoptosis (Patel et al. 2019).

Herein, different hydrogel-based constructs as carrier for various types of drugs or active biomolecules will be addressed. Fan et al. demonstrated that hydrogels with KGN-conjugated polyurethane nanoparticles and TGF- β 3 is effective in cartilage regeneration. KGN and TGF- β 3 have synergistic effects on chondrogenesis of MSCs (Fan et al. 2020). Another research used KGN-loaded hydrogels for treatment of defective cartilage and introduced it as a proper composite for tissue engineering. Researchers developed constructs composed of chitosan hydrogel and polycaprolactone mat. The carboxyl groups in KGN bonded to the amine groups of chitosan which would assist in its prolonged exposure. The results of this study showed that sustained release of KGN from construct could promote MSC differentiation. According to the immunofluorescent, histological staining and molecular analysis, the expression of chondrogenic markers as Sox-9, collagen type II and

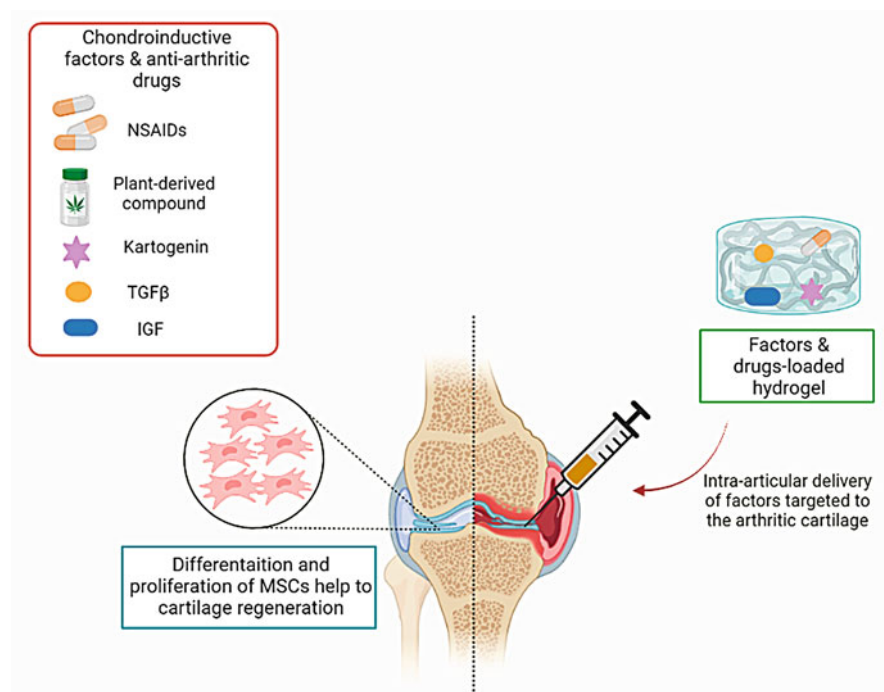


Fig. 10.2 Hydrogels as convenient vehicles for controlled delivery of chondroinductive factors. Chondroinductive factors and anti-arthritis drugs as NSAIDs can be loaded in an injectable hydrogel and desirable differentiation and cell growth of MSCs and cartilage reconstruction will be occurred

aggrecan were higher in the presence of KGN (Houreh et al. 2021). Shi et al. provided an innovative therapeutic structure by KGN-loaded poly (lactic-co-glycolic acid) (PLGA) nanoparticles integrated with photo cross-linked hydrogel. In vivo administration of this platform resulted in cartilage regeneration and forming hyaline cartilage. In addition, combination of KGN, BMP-2, and TGF-β3 upregulated the chondrogenesis markers like sox-9 and Col II in the human MSCs (Shi et al. 2016).

It has been demonstrated that co-delivery of IGF-1 and adipose-derived MSCs represents synergistic therapeutic effects on osteochondral defects. In this regard, Cho et al. applied dual delivery of IGF-1 and MSCs by designing thiolated gelatin/poly(ethylene glycol) diacrylate interpenetrating network hydrogels containing tertiary complex of poly(ethylene arginylaspartate diglyceride) polycation, heparin, and cargo IGF-1. The developed construct resulted in a functional 3D model to induce cartilage regeneration and differentiation of MSCs in vivo. Notably, sustained release of IGF-1 caused upregulation of chondrogenic markers in vitro (Cho et al. 2020). Some studies have focused on improving injectable hydrogel composites in order to achieve the best functional IA delivery system. Due to the poor localization and short maintenance of the hyaluronate (HA)-based hydrogels

and fast degradation of this composite in the articular cartilage, Feng et al. constructed sulfated HA hydrogel and improved delivery of TGF- β 1 in vitro and in vivo. They indicated that sulfation could induce chondrogenesis of MSCs, ameliorate efficiency of TGF- β 1 binding to HA-based hydrogel, and improve its availability within hydrogel. Moreover, degradation of sulfated HA hydrogels by hyaluronidase enzyme was slower than HA hydrogels (Feng et al. 2017).

In order to design a composite construct for long-term exposure of growth factor, a collagen-based hydrogel containing graphene oxide nanoparticles as a carrier of TGF- β 3 was fabricated. This composite increased the stability of the growth factor and its sustained release was occurred. Thereby, higher potency of this delivery system led to appropriate chondrogenic differentiation of MSCs (Zhou et al. 2019). Li et al. evaluated the effect of BMP-7 and TGF- β 1 on cartilage repair. They constructed composite structure of TGF- β 1-loaded chitosan nanoparticles and silk fibroin hydrogels incorporated with BMP-2. This structure with high biocompatibility showed chondrogenesis ability by increasing the expression of Collagen II, aggrecan, and β -catenin in vitro. Additionally, in vivo experiments exhibited that these scaffolds could successfully regenerate the cartilage defects (Li et al. 2021b). A novel type of dual network hydrogel which contains silk fibroin and alginate-polyoxamer including BMP-7-loaded hyaluronate-based nanoparticles has been built for cartilage tissue repair. Gene expression and proliferation analysis indicated high chondrogenic differentiation of synovium-derived MSCs (Min et al. 2020).

Moreover, plant-derived compounds could be loaded into hydrogels. Quercetin (Que) is a flavonoid compound with anti-inflammation and chondrogenesis effects. One research incorporated Que into a well-designed injectable hydrogel system for cartilage formation in vivo. Que-loaded injectable sodium alginate/bio glass hydrogel released in a controlled manner and its bioavailability was enhanced. Besides, this promising drug delivery system altered the defective cartilage to normal chondrocyte phenotype by inducing expression of Sox-9, aggrecan and Col II. Also, it reduced inflammation by promoting polarization of macrophage M2. ECM degradation was inhibited by lower expression of MMP-13 and MMP-1 (Yu et al. 2019). Another plant-derived compound with anti-inflammatory and anti-carcinogenic effects is sulforaphane (SFN) which is extracted from broccoli. According to the results of one study, SFN-loaded polyoxamer-hyaluronate hybrid hydrogel could diminish the IL-1 β expression and promote Col II expression in a damaged cartilage in vitro and ex vivo (do Nascimento et al. 2021).

As NSAIDs have side effects on gastrointestinal tract, targeted delivery of them is recommended. Garcia-Fernandez et al. have proposed semi-interpenetrating network hydrogels composed of gelatin and hyaluronate. In this study, naproxen and dexamethasone with anti-inflammatory properties were incorporated into the injectable semi-IPN network and tested in vitro and in vivo. The results revealed not only reduced inflammation and production of MMPs but also upregulation of collagen II in an injured cartilage. Due to non-toxic structure of semi-IPN hydrogels, they could help to chondrocytes viability (García Fernández et al. 2020). Meloxicam is the other type of NSAIDs that its IA delivery is beneficial. Meloxicam-loaded nanoparticles encapsulated in an injectable carboxymethyl chitosan-methylcellulose-pluronic

hydrogel indicated sustained release of meloxicam, biocompatibility, and enhancement of cell proliferation. In addition, this platform of meloxicam delivery could significantly reconstruct cartilage defect (Fattahpour et al. 2020).

In summary, IA delivery of chondroinductive growth factors, NSAIDs, and plant-derived compounds via hydrogels is a non-invasive therapeutic method which could help to decrease side effects of NSAIDs. Due to the similarity between hydrogels and ECM structure, they are also promising candidates for cartilage regeneration. This novel remedial field could pave the new way in treatment of some diseases as OA. However, further researchers are required for identification of more functional growth factors and evaluation of their mechanisms which happen during IA delivery process.

10.4 Bioprinting of Hydrogels for Cartilage Regeneration

Three-dimensional (3D) bioprinting is an emerging technology with capability to develop complicated constructs in order to resemble the heterogeneous structure of defective tissues. Layer-by-layer deposition of cell-laden bioinks with micrometer precision in this approach can excel the traditional scaffold preparation methods due to the more precisely control over printed structure and cell distribution. The bottom-top approach in bioprinting technology can achieve constructs with high cell density along with superior cell–cell communication and desirable cell–ECM interaction which may promote tissue reconstruction. Hydrogels with capability of desirable injectability and shear-thinning properties for controlled deposition of cells and biomolecules as well as their intrinsic biocompatibility, high water content, and structural similarity to extracellular matrix may be the best candidate as printed bioinks (Fig. 10.3) (Unagolla and Jayasuriya 2020; Daly et al. 2017). To date, various types of hydrogels have evaluated as printed bioinks for cartilage regeneration.

In an attempt to design an appropriate bioink for cartilage reconstruction, combination of nanofibrillated cellulose with excellent shear-thinning capabilities and alginate with rapid cross-linking ability was utilized. Both 2D grid-like structures and 3D constructs could be fabricated due to the proper shear-thinning behavior of developed bioinks. The outstanding characteristics such as shear thinning, shape fidelity, and printing resolution revealed the priority of nanofibrillated cellulose before cross-linking, whereas the alginate was dominated by improving the mechanical stability of construct after cross-linking. The printed bioink could successfully form anatomically shaped cartilage structures, such as human ear and sheep meniscus with desirable cytocompatibility in contact with human chondrocytes (Markstedt et al. 2015). In a study for preparing a 3D printed biphasic construct for cartilage repair, the top layer of cell-encapsulated biodegradable gelatin methacrylate (GelMA) hydrogel was located on the supporting layer composed of macroporous poly(ϵ -caprolactone) (PCL) scaffold. The biphasic construct was seeded by co-culture of bone marrow-derived MSCs and costal chondrocytes. The PCL/GelMA biphasic scaffolds represented desirable cartilage regeneration

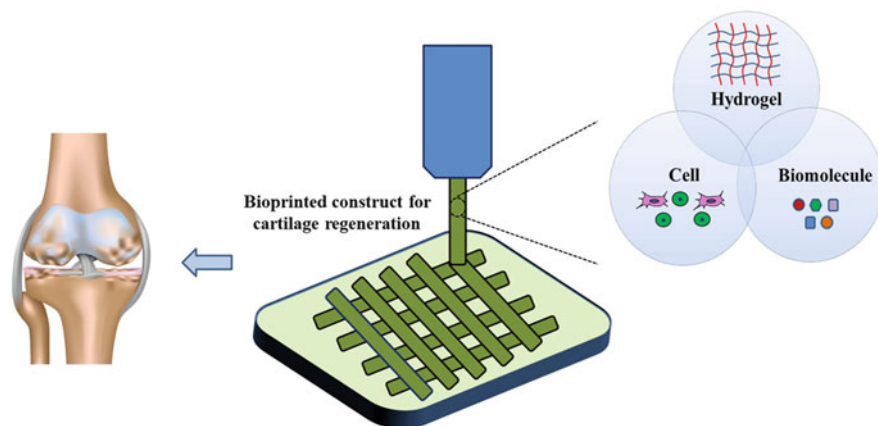


Fig. 10.3 Bioprinting of hydrogels for cartilage regeneration. Hydrogels with appropriate injectability and rheological features can be promising candidates as printed bioink for controlled deposition of cells and biomolecules

following 12 weeks implantation in a rat osteochondral defect model and promoted the mechanical properties of engineered construct proportional to that of native cartilage. However, the synergistic effect of MSCs and chondrocytes was adequate for appropriate chondrogenesis; cartilage repair was superior under TGF- β 3 incorporation (Cao et al. 2021).

A macroporous hydrogel scaffold was fabricated through extrusion-based low-temperature 3D printing. The hydrogel was fabricated by horseradish peroxidase (HRP)-mediated cross-linking of silk fibroin and tyramine-conjugated gelatin and exhibited outstanding structural stability as well as adjustable mechanical properties and degradation rate, proportional to native cartilage. Human adipose-derived MSCs were inoculated into hydrogel in two forms of suspension and aggregate. The internal structure of the hydrogel was suitable for complete retention of cell aggregates. The cell aggregate seeding resulted in proper differentiation of MSCs into hyaline cartilage, whereas cell suspension seeding resulted in fibrocartilage formation. The results of *in vivo* studies in a rabbit cartilage defect after 12 weeks demonstrated that hydrogel combined with cell aggregates improved articular cartilage regeneration significantly (Li et al. 2021c). In order to develop a 3D-printed hydrogel composed of platelet-rich plasma (PRP) and gelatin methacryloyl (GelMA), digital micro-mirror device (DMD) technique was utilized. The differentiation potential of bone marrow-derived MSCs and macrophage polarization was evaluated within GelMA hydrogels containing different PRP concentrations. The hydrogels with 20% (w/w) PRP illustrated convenient proliferation and migration as well as chondrogenic differentiation of MSCs. Moreover, macrophages were significantly polarized into M2 phenotype within optimized hydrogel as revealed by high expression of M2 markers such as Arg1 and CD206. The GelMA hydrogel containing 20% (w/w) PRP represented more cartilage and

subchondral bone repair in comparison with pure GelMA hydrogel (Jiang et al. 2021).

In an attempt to design a printable self-healing polysaccharide-based hydrogel for cartilage regeneration, partially oxidized hyaluronate (OHA) was combined with glycol chitosan (GC) in the presence of adipic acid dihydrazide (ADH). Incorporation of two different dynamic bonds within hydrogel resulted in appropriate self-healing ability of OHA/GC/ADH hydrogel. These dynamic bonds consisted of imine bonds formed by Schiff base reaction between OHA and GC, as well as acylhydrazone bonds obtained via the reaction between OHA and ADH. The concentrations and molecular weights of the constituent polymers were significantly affect the flow and mechanical properties of the self-healing hydrogels. The prepared 3D construct exhibited outstanding structural stability, without requirement for applying supporting materials or secondary cross-linking procedure. The OHA/GC/ADH hydrogel exhibited proper biocompatibility and chondrogenic differentiation of ATDC5 cells. Moreover, the printing process did not induce any adverse effect on expression of chondrogenic marker genes such as SOX-9 and collagen type II in ATDC5 cells encapsulated within hydrogel (Kim et al. 2019). Personalized scaffolds with biomimetic gradient structure were developed by incorporating mesoporous silicate-doped calcium phosphate cement (MS/CPC) within hydrogel composed of hydroxybutyl chitosan (HBC) and oxidized chondroitin sulfate (OCS). Optimizing the concentration of OCS could improve proliferation, adhesion, and ECM formation of chondrocytes. Utilizing MS/CPC with a bioactive surface similar to bone tissue could promote osteogenesis and vascularization properties. 3D printed personalized construct for cartilage-subchondral repair was successfully obtained based on the self-cross-linking properties of the Schiff-based HBC/OCS hydrogel and the macroporous structure of MS/CPC scaffolds. Hydrogel containing 20 mg/mL OCS could promote the proliferation, adhesion of chondrocytes, Col II and SOX-9 gene expression as well as articular cartilage ECM formation. Furthermore, hydrogel comprising 0.5 mg/mL OCS illustrated anti-angiogenic properties similar to natural cartilage, which significantly prevented migration and tube formation of endothelial cells (Li et al. 2020b). A biohybrid construct with gradient structure was developed by printing poly (N-acryloylglycinamide) (PNAGA)-based hydrogel by controlled distribution of TGF- β 1 and β -tricalcium phosphate (β -TCP) in separate layers. The hydrogel composed of poly [N-acryloylglycinamide-co-N-[tris(hydroxymethyl) methyl] acrylamide (NAGA-co-THMMA)] copolymer demonstrated temperature sensitive reversible gel to sol transition and shear-thinning behaviors. Copolymerization of THMMA could relieve the hydrogen bonding interactions of PNAGA and subsequently decline the extrusion temperature and improve the stabilizing swelling of the hydrogel. Thermal-assisted extrusion printing technique was applied for designing bilayer gradient hydrogel containing TGF- β 1 on the top layers and β -TCP on the bottom layers. The resultant gradient scaffold represented appropriate biocompatibility, high interconnected porosity, and desirable mechanical characteristics as well as high resolution. The incorporation of β -TCP could significantly promote the physicochemical properties of construct and also improve the proliferation, ALP

activity, and osteogenic differentiation of MSCs. Moreover, introduction of TGF- β 1 resulted in desirable growth and chondrogenic differentiation of the MSCs. *In vivo* studies demonstrated that biohybrid gradient hydrogel could simultaneously improve the repair of both cartilage and subchondral bone within osteochondral defects (Gao et al. 2018).

Taken together, bioprinting technology, by precise deposition of appropriate hydrogel-based bioinks comprising cellular and chondroinductive components could construct a biomimetic microenvironment for resembling the complex architecture of native articular cartilage. One of the main capabilities of bioprinting approach is desirable reconstruction of anisotropic osteochondral tissue with outstanding functional performance. Advancements in biomaterials technology and development of stimuli-responsive hydrogels can introduce promising substitutes for resembling time-dependent morphological tissue changes. The tissue mimicking bioprinted constructs with ability to organization at cell-scale resolution may result in convenient articular reconstruction.

10.5 Conclusion and Outlook

We reviewed recent advances in developing hydrogel-based constructs for cartilage reconstruction in terms of cross-linking strategies for hydrogel preparation as well as their applications as delivery systems and bioprinted constructs. Artificial ECM-mimicking hydrogels can be fabricated through different cross-linking approaches such as physical and chemical conditions. Despite the beneficial features of both types of hydrogels, inadequate mechanical stability of physically crosslinked hydrogels as well as brittleness and harsh preparation condition of chemically cross-linked hydrogels faced these approaches with major challenges. Thereby, double-network hydrogels possess useful characteristics of physically and chemically cross-linking hydrogels as well as desirable self-healing, toughness, and recoverability and may be a promising substitute for load-bearing articular cartilage. Despite extensive advances of hydrogels in the field of cartilage regeneration, their translation into clinical setting has been under progress due to the numerous challenges. In this way, well-integration of implanted construct in defective region to surrounding host tissue, adjusting the degradation rate to match tissue regeneration and providing the sufficient mechanical strength as load-bearing support are the major challenges. For achievement of desirable tissue regeneration, hydrogels should promote cell recruitments and dynamic extracellular signaling.

Designing personalized osteochondral constructs proportional to irregular shapes and multiple dimensions of defects along with appropriate integration with host tissue should be considered.

Development of gradient constructs including individual sections proportional to soft cartilage and hard bone tissue as well as soft-hard interfaces is the optimal option for reconstruction of osteochondral tissue. By incorporation of chondrogenic and osteogenic cells as well as chondroinductive agents within hybrid gradient structures, functional osteochondral substitutes can be formed. Advanced

manufacturing techniques such as bioprinting can successfully provide robust printed bioinks with gradient composition and organized zonal architecture, similar to the structure of osteochondral tissue. By progress in automated and computerized arrangement of cell-laden constructs comprising appropriate bioactive molecules, next generation of personalized therapeutics can significantly promote human cartilage reconstruction. Altogether, accurate selection of convenient hydrogel-based constructs with desirable regenerative capabilities and mechanical features as well as comprehensive evaluations in pre-clinical trials can assist in a successful translation to the clinic setting.

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Shape-Memory Polymers in Cartilage Tissue Engineering 11

Parisa Zadehnajar, Babak Akbari, Amirabbas Amini,
and Lobat Tayebi

Abstract

This chapter is dedicated to discussing the possibility and advantageous of using shape-memory polymers in cartilage tissue engineering by providing a supportive scaffold for the regeneration of articular cartilage tissues. Shape-memory polymers are a kind of polymers that can be unlimitedly changed between a permanent and a temporary shape. They have been considered as adaptable candidates to make suitable devices for minimally invasive implantation. These devices can be fixed to a temporary compact state for convenient operation, and they will automatically restore their permanent predetermined shape after implantation. Besides the shape-memory properties, biodegradability can add a fascinating capability to such polymers that can be useful for cartilage tissue engineering. The main advantage of biodegradable shape-memory polymers for cartilage tissue engineering is preventing a second surgery for removing the construct while have the ability to restore their shape while under pressure or loads. These polymers are an emergent class of polymers that facilitate the regeneration process of natural tissues including cartilage.

P. Zadehnajar · B. Akbari (✉)

Life Science Engineering Department, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran

e-mail: babakbari@ut.ac.ir

A. Amini

Department of Materials Engineering, Science and Research Branch, Islamic Azad University, Tehran, Iran

L. Tayebi

Marquette University School of Dentistry, Milwaukee, WI, USA

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M. Baghaban Eslaminejad, S. Hosseini (eds.), *Cartilage: From Biology to Biofabrication*, https://doi.org/10.1007/978-981-99-2452-3_11

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Keywords

Shape-memory polymers · Biodegradable polymers · Articular cartilage · Tissue engineering · Hydrogels

11.1 Introduction

Shape-memory polymers (SMP) are a unique group of mechanically active or intelligent materials fabricated from natural or synthetic raw polymers. These polymers' high deformability and stimuli-responsiveness enable them to transform from a transient shape to a preset shape in response to the presence of the correct external stimulus. Polymers do not naturally possess the shape-memory effect; the specific processing steps and conditions that must be met. Entropic elasticity is the foundation of its mechanism (Delaey et al. 2020). One of the most strongly coiled conformations of an amorphous polymer chain is the one that is most favorable entropically. The random coils will elongate when elastic deformation takes place in an amorphous polymer, aligning them along the direction of the applied stress and lowering the number of potential configurations and, as a result, the entropy (Delaey et al. 2020). Since a polymer's chain entanglements operate as a form of physical cross-link because their molar mass is sufficiently large, they impede the mobility of the polymer chains. As a result, when the tension is released, the polymers revert to their original, more entropically attractive coiled configuration (Delaey et al. 2020). It might be argued that the polymer's "memory" contains a record of its original, unreformed condition (Delaey et al. 2020).

A kind of polymer known as SMP may be transformed countless times between a permanent and temporary shape. Under particular circumstances, such as heating beyond the glass transition or melting temperatures, a transient shape can be obtained by mechanical deformation that is then fixed while the deformation is still in place. Fixation methods include crystallization, chemical cross-linking, and supramolecular interactions. In reality, the polymer will hold its temporary form when tension is released until an appropriate external trigger is supplied, such as light, temperature, chemical agents, etc. As a result, the original form will be restored (Delaey et al. 2020).

The shape-memory effect can be thermodynamically linked to the release and storage of strain energy by consuming network entropy. As the polymer temperature is increased to T_{Trans} , an increase in the entropy leads to the activation of the polymer to a high energy state with indefinite chain mobility of the switching segments (Ramaraju et al. 2020). When the deformation occurs in the polymer toward the programmed shape and is cooled, the switching segments lock the polymer in an unstable state, which does not release the strain energy. Cooling the programmed polymer in an unstable energy state makes it kinetically undesirable for the switching segments to return to their original state. On the other hand, heating to T_{Trans} makes the polymer regain its chain mobility and provides sufficient kinetic energy due to a

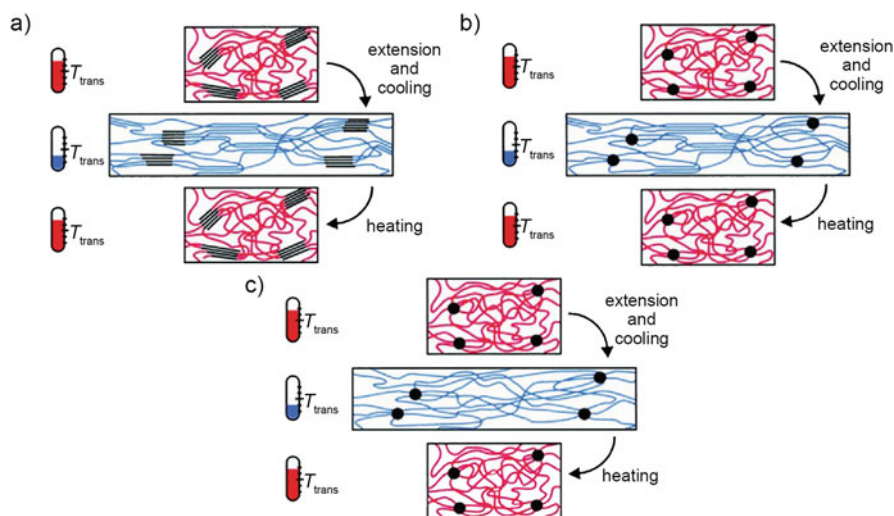


Fig. 11.1 Changes in polymer networks during one-way shape-memory cycles indicate (a) thermal transitions in a multiblock copolymer $T_{trans} = T_m$, (b) indicating thermal changes in a covalently cross-linked crystalline polymer $T_{trans} = T_m$, (c) indicating network structure during the transition of a glassy polymer $T_{trans} = T_g$ where the flexible transitional portions were T_{trans} in red and cooled portion below T_{trans} in blue. Obtained from reference (Ramaraju et al. 2020) with permission

raised entropy. This allows the switching segments to release the stored strain energy and recover back to its original shape (Fig. 11.1) (Ramaraju et al. 2020).

In general, the SMPs can be programmed to a temporary shape and then returned to their original shape by applying an appropriate external stimulus such as heat, moisture, pH, light, electricity, alternating magnetic field, radiation heating, laser heating, microwaves, pressure, solvent, solvent vapors, etc. (Sarvari et al. 2022). Temperature and light have been the two primary direct triggers mentioned in literature. Nevertheless, other suggested indirect causes include exposure to a solvent, ultrasound, and more (Delaey et al. 2020).

This chapter will explain the SMP as a biomaterial and how it can be used for biomedical applications. The biodegradable capability of SMP will be discussed in detail. After general descriptions about tissue engineering and more specifically cartilage tissue engineering, using SMP in cartilage tissue engineering scaffolds will be discussed.

11.2 Shape-Memory Polymer (SMP) as a Biomaterial

Nontoxicity is the most prominent feature of a carefully designed biomaterial; it refers to no presence of carcinogenic, allergic, inflammatory, or blood-compatible materials. A nontoxic biomaterial should not release or generate any molecules unless it is specifically designed to do so (Delaey et al. 2020). Biocompatibility is another essential feature of biomaterials. It refers to a biomaterial's capacity to function in a particular application with the proper host reaction (Delaey et al. 2020). Utilizing biopolymers instead of synthetic polymers is beneficial because the former often causes fewer inflammatory responses.

Additionally, the mechanical characteristics of the implant material should be compatible with the qualities of the surrounding tissue. Table 11.1 compares reported shape-memory polymers to the summary of the module of several human tissues. Additionally, it can signify choosing a shape-memory polymer for biological purposes (Delaey et al. 2020). Poly (diolamine), poly (glycolic acid), poly (lactic acid), and poly (caprolactone) are examples of synthetic polymers with shape-memory properties, which have been used tremendously in biomaterials (Delaey et al. 2020). Table 11.2 shows an overview of shape-memory polymers.

Table 11.1 Young's moduli of various human tissues and reported shape-memory polymers are compared (Delaey et al. 2020)

Tissue/material	Modulus (MPa)	Ref
Femur	2×10^3 – 19.3×10^3	Keller et al. (1990)
Tendon	1.2×10^3	Maganaris (2002)
Muscle	1.45–20	Deguchi et al. (2006); De Winkel et al. (1993)
Spinal cord and gray matter	2	McKee et al. (2011)
Electrospun poly(p-dioxane) and poly(ϵ -caprolactone) networks	3.5–5	Kratz et al. (2011)
Poly(3-hydroxybutyrate)- <i>co</i> -(3-hydroxyvalerate)	100.1	Kim et al. (2005)
Chitosan	4246	Kim et al. (2005)
Oligo-(ϵ -caprolactone) dimethacrylate and <i>n</i> -butyl acrylate	0.5–71	Rickert et al. (2003); Lendlein et al. (2001)
Methyl methacrylate- <i>co</i> -polyethylene glycol methacrylate	9.3–23	Yakacki et al. (2008)

Table 11.2 The summary of shape-memory polymers and their uses (Delaey et al. 2020)

Chemical composition	Trigger	Form	Application	Ref
Poly(ϵ -caprolactone)-based polyurethane	Ultrasound	2D shapes	Drug delivery	Han et al. (2013)
Oligo(ϵ -caprolactone) diol + oligo(p-dioxanone) diol + diisocyanate	Thermal	Monofilament	Sutures	Lendlein and Langer (2002)
Poly(urethane)	Photothermal	Foam	Aneurysm occlusion	Maitland et al. (2007)
Poly(ϵ -caprolactone)acrylate + hydroxyapatite nanoparticles	Thermal	2D shapes	Bone defect repair	Tian et al. (2019)
Poly(glycerol sebacate) + poly(1,3-propylene sebacate) + kartogenin	Thermal	2D films foam	Cartilage regrowth	Xuan et al. (2020)

11.3 Shape-Memory Polymers in Biomedical Applications

The unique characteristics of shape-memory polymers give them the potential to be widely used in medical applications. Some of these applications are discussed in this section.

11.3.1 Polymers with Shape Memory for Delivery of Drugs

Shape-memory polymer use is significant in drug delivery. Since the shape-memory effect may readily be employed to immobilize the drug delivery device at the target region without external manipulation, the combination of drug delivery with shape-memory has gained substantial interest (Delaey et al. 2020). It has been claimed that adding pharmaceuticals to a co-poly(ester-urethane) SMP does not appreciably reduce the material's capacity for shape memory. So, shape-memory polymers may be used to create drug-release targets in watery conditions. In the case of a drug-releasing shape-memory polymer based on poly (methyl methacrylate-co-butyl acrylate), the drug release may be halted at any moment, in addition to having the capacity to partially regain shape (Delaey et al. 2020).

11.3.2 Shape-Memory Polymers for Cardiovascular

Shape-memory polymers can be used in the field of cardiovascular medicine. Polymeric shape-memory stent restenosis is reduced because of the mechanical similarities between polymeric materials and the vascular epithelium. A study team created a less invasive functioning tissue patch made of poly (octamethylene maleate (anhydride) citrate). Without impacting cell viability, the patch may be introduced through an aperture and then expanded in vivo to its original form (Delaey et al. 2020). Open-heart surgery, generally required for injuries comparable to this, can take its place (Montgomery et al. 2017).

11.3.3 Polymers with Shape Memory and Antibacterial Properties

SMP will decrease the likelihood of infection and improve the material's suitability for biomedical uses. They do not have intrinsic antibacterial properties and require additives to give them an antibacterial effect. These additives can also enhance other properties (Delaey et al. 2020). Silver nanoparticles were bonded to cellulose nanocrystals in a PCL-based shape-memory network in research on light-responsive SMPs. The antibacterial activity in the polymeric matrix is due to the silver nanoparticles (Toncheva et al. 2018). This technique provides an interesting opportunity to obtain a photo-responsive SMP modified with antibacterial properties by selecting an appropriate additive.

11.4 Biodegradable Shape-Memory Polymers (BSMP)

Biodegradable polymer SMPs are attractive due to the increased environmental concerns. The bio-based materials can be synthesized from different sources such as microorganisms (polyhydroxyalkanoates (PHAs)), diverse polymerizations (polylactic acid (PLA)), or biomass (starch). In addition, some widely used biodegradable polymers have a petroleum-based origin, such as poly (ϵ -caprolactone) (PCL) and several aliphatic polyesters (Zheng et al. 2017; Tian et al. 2012). Aside from their shape-memory properties, biodegradability presents an interesting field on biodegradable shape-memory polymers (BSMPs). The main advantages of BSMPs are the ability to restore their shape related to their shape-memory feature and prevent a second surgery for removal since the device will be degraded in the body. Exposure to biological fluid and/or tissues starts their biodegradation process (Peterson et al. 2017).

Due to their unique characteristics, shape-memory materials, specifically nickel-titanium alloys, have potential applications in various mini-invasive clinical procedures. This shows significant improvements in clinical outcomes and standard of care compared to open procedures. However, tissue engineering approaches have attracted significant interest due to concerns regarding erosion events, device failure, and consequent morbidities.

These approaches can support or stimulate the patient's tissue regenerative processes to successfully regenerate the tissue. In addition, growing efforts in addressing the challenges related to SMPs have led to the rapid development of a novel class of materials called biodegradable shape-memory polymers.

Compaction and delivery through keyhole incisions, expansion after reaching the target tissue, maintenance of mechanical continuity with surrounding tissues after implantation, attachment of regenerative cells which cause growth and infiltration, and degradation into nontoxic byproducts are positive features that have led to the fabrication of biodegradable SMPs (Ramaraju et al. 2020). Polyesters are mainly used for the fabrication of shape-memory polymers with biodegradability properties because the biodegradation of these materials proceeds via hydrolysis. Homopolymers, copolymers, terpolymers, block copolymers, and interpenetrating networks are examples of biodegradable shape-memory polymers that have extensive applications.

Polycaprolactone (PCL), poly(lactic-co-glycolic acid) (PLGA), polylactic acid (PLLA), polyurethanes (PUs), and polycarbonates have been considered for various polycarbonates that were studied for several medical purposes, including scaffolds for craniofacial reconstruction, orthopedics, antibacterial agents, periodontal healing, and bone regeneration (Fig. 11.2) (Sun et al. 2017).

The mechanical properties of these materials used in biomedical implants are outside the range of the required mechanical properties related to most biological soft tissues. They need considerable adjustment to address hard tissue pathologies (Ramaraju et al. 2020).

BSMPs are an emergent class of polymers that facilitate the regeneration process of natural tissues since they can be replaced by the patient's tissue during tissue

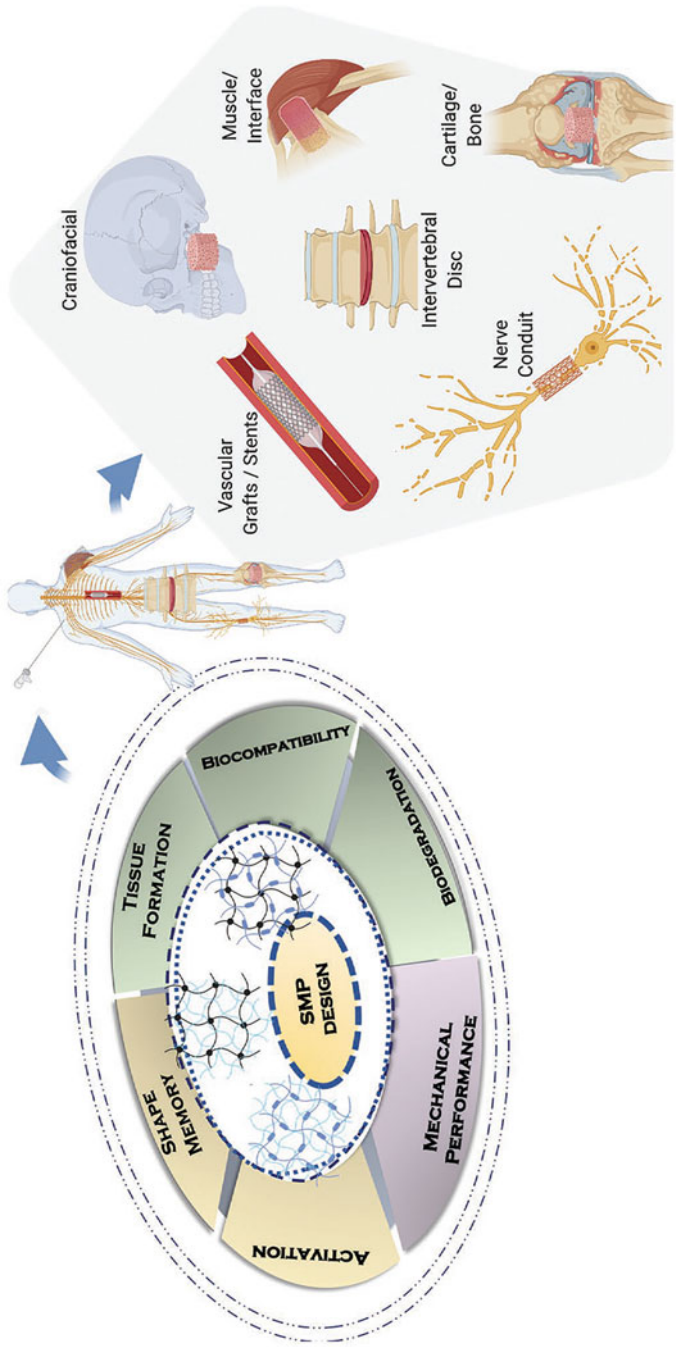


Fig. 11.2 Shape-memory design and design inputs in the context of tissue repair in diverse uses of tissue engineering. Obtained from reference (Ramaraju et al. 2020) with permission

repair. Most biomedical SMPs use temperature as the triggering cue and adjust their transition temperature (T_{Trans}) at or below body temperature (37 °C). Polymers with transition temperatures above 37 °C will need to be heated during delivery or subjected to a solution-mediated activation (Rodriguez et al. 2014; Baer et al. 2007). Temperature is a prevalent strategy for shape-memory activation for SMPs used experimentally in clinical applications (Ramaraju et al. 2020).

11.5 Tissue Engineering

Diseases, injuries, and traumas are responsible for tissue damage in the human body. The low availability of conventional tissue autografts and allografts increases the need to regenerate these damaged tissues through a manner called tissue engineering (Peterson et al. 2017).

Tissue engineering is a novel regenerative approach in the medical field which has attracted researchers' attention since 1990. It is a useful way of mending the tissue's function without the donor organ's immunological response or the grafting tissue's instability. Tissue engineering is a cooperative principle between materials, cells, and growth factors (Mirmusavi et al. 2019; Zadehnajar et al. 2019a, b). Materials can provide a supportive area scaffold for cell attachment, differentiation, proliferation, and migration (Zadehnajar et al. 2019a; Mirmusavi et al. 2022). They can mimic the functional and biological features of the natural cartilage tissue. Different characteristics of scaffolds should be considered in the engineering of cartilage tissue order to fulfill the standards such as biocompatibility, similar mechanical properties to native tissue, controllable degradation rates, cell encapsulation capacity, drugs and genes delivery, easy handling, osteoconductive potential, and good flexibility (Eslahi et al. 2016; Lee and Shin 2007; Hutmacher 2000).

To regenerate functional tissues, the tissue engineering paradigm focuses on the combination of scaffolds, cells, and signals. Due to the ability of bioactive materials to function properly as scaffolds, they provide structural and mechanical support, and deliver bioactive signals to direct cellular differentiation and tissue growth (ECM), they have been developed extensively. One of the most promising alternative therapies for articular cartilage abnormalities has been demonstrated to be tissue engineering (Iwasa et al. 2009; Lee et al. 2003). One of the most crucial components of this approach is a biodegradable, three-dimensional porous scaffold that can accept the transplanted cells as a supporting matrix and direct the development of new tissue. To provide acceptable scaffolds for cartilage tissue engineering applications, synthetic polymers that are naturally produced and biodegradable are frequently employed (Dai et al. 2010).

A well-designed tissue engineering scaffold can simulate the extracellular matrix (ECM) of natural tissue, provide an appropriate environment for cell survival and regulate cell behavior and function. In addition, it is a three-dimensional porous structure with interconnected porosity, biocompatibility, and biodegradability for biomimicry of natural ECM (Mirmusavi et al. 2019, 2022; Zadehnajar et al. 2019a, b; Mohammadalizadeh et al. 2019). The main goal of tissue engineering is

not to replace the tissue with artificial material but to regenerate the natural tissue. Therefore, scaffolds must be biodegradable to be eliminated from the body environment during the gradual tissue growth and replaced with new-regenerated tissue. In other words, there should be a balance between tissue regeneration and biodegradation rates (Mirmusavi et al. 2019; Zadehnajar et al. 2019a, b; Mohammadalizadeh et al. 2019).

Biodegradable shape-memory polymers (BSMP)-based scaffolds have been developed for different tissue regenerations such as cartilage, nerve, or vascular tissue; however, bone tissue regeneration is the main application of these materials. The shape-memory capability of these materials could modify the surface topography allowing control over cell orientation, growth, and differentiation (Peterson et al. 2017).

The shape-memory behavior of biodegradable sutures has made them remarkable in tissue engineering. This advantage produces self-tightening properties. These sutures can disintegrate after the wound has healed, eliminating the necessity for suture removal (Delaey et al. 2020). This enables doctors to initiate the shape-memory effect after gently suturing a wound to help seal it (Delaey et al. 2020).

It is reported that the microgrooves created on BSMP surfaces could affect aligned cell culturing. When the shape recovery is triggered, the anisotropic topography will be switched to a flat, featureless surface. This loss of topography reduces the cell alignment without decreasing its viability (Neuss et al. 2009). A study team used the scaffold of shape-memory poly (ε-caprolactone) dimethacrylate. On this scaffold, human mesenchymal stem cells, L929 murine fibroblasts, human mesothelial cells, and rat mesothelium cell activities were examined to assess their appropriateness for medicinal applications. The findings demonstrated that the polymer maintained mesenchymal stem cells' ability to differentiate, and most adherent cells were unaffected by shape-memory effect activation (Neuss et al. 2009).

Repairing bone and cartilage defects is another possible application of biodegradable shape-memory polymer scaffolds (Peterson et al. 2017). The capability of BSMP to adapt their shape makes them interesting materials for repairing irregular bone and cartilage defects. A research group fabricated PCL-based surfaces patterned with nano grooves. They can switch their orientation at 32–37 °C. This reorganization can regulate cellular differentiation, maturation, and function. In this case, the shape transition is triggered via temperature and induces a variation of 90° on the nanogroove pattern. This variation can make modifications to the contractile direction and structural organization of cardiomyocyte sheets (Mengsteab et al. 2016). Shape-memory scaffolds made by solvent casting were mentioned in a paper (Zhang et al. 2014). They repaired uneven cranial bone lesions with poly (ε-caprolactone)-poly(L-lactic acid) semi-interpenetrating networks. These biodegradable structures successfully fit irregular defects and improve osteointegration (Zhang et al. 2014).

11.6 Cartilage Tissue Engineering

Traumas, injuries, and joint disorders such as osteoarthritis lead to cartilage degeneration and disability. Cartilage has a limited reparative and regenerative capacity because of its avascular nature. Currently, surgery is the most common procedure to treat cartilage degeneration, which often leads to the formation of fibrous repair tissues followed by impaired function (Keeney et al. 2011). Intense research has been conducted to support cartilage tissue regeneration and address critical medical demands. The most effective FDA-approved method for regenerating cartilage abnormalities is called MACI®, which involves matrix-applied implantation of autologous chondrocytes cultivated on a membrane made of porcine collagen (He et al. 2021). This therapy does have certain restrictions, however. First, collagen types I and III from swine tissue are present in the membrane used for cell culture, which raises the possibility of an immunological reaction following implantation. Second, it necessitates two surgical procedures: a mini-arthrotomy for implantation and arthroscopy for chondrocyte collection. Therefore, we will be able to enhance this therapy and make it attractive to repair cartilage abnormalities by reducing the number of procedures or avoiding surgical implantations while enhancing biocompatibility (He et al. 2021).

Articular cartilage (AC) is a soft layer of specialized connective tissue with viscoelastic properties. It covers the bone surface in joints and provides a lubricating surface, which can tolerate high loading for a long time. This transfer of the minimum pressure toward underlying subchondral bones absorbs shock, decreases friction, and facilitates joint movements during daily activities. Figure 11.3 demonstrates the schematic of the macro and microstructure of articular cartilage (Farokhi et al. 2020). Although several surgical and nonsurgical treatments are available, repairing adult AC remains a challenge because of its limited potential for satisfactory regeneration. The tissue's capacity to be regenerated depends on vascularization potential, the amount of cell-to-matrix ratio, age, and the size of the structural damage (Frenkel and Di Cesare 2004; Vinatier et al. 2006). An injured AC in the superficial zone needs time to be repaired. In addition, the expansion of injuries to the subchondral bony plate in some cartilage diseases, such as osteoarthritis (OA), is responsible for immobility, pain, and joint breakdown (Fiorica et al. 2015). The process of cartilage regeneration leads to the formation of fibrocartilage tissue, which is not the same as the native tissue in terms of biological and mechanical properties and destroys the undamaged tissue. Therefore, it is necessary to find an alternative, innovative, and therapeutic approach to cartilage treatment (Farokhi et al. 2020).

It is generally considered that articular cartilage has a very limited potential for a spontaneous healing process, whether the damage is caused by degenerative alterations and pathological conditions, or traumas such as sports injuries. Even minor cartilage lesions might lead to progressive degeneration and damage (Dai et al. 2010). This poor intrinsic repair capacity corresponds to the avascular structure of cartilage that consists of chondrocytes sparsely embedded in an organic matrix (Nerem and Sambanis 1995; Magne et al. 2005). Subchondral drilling,

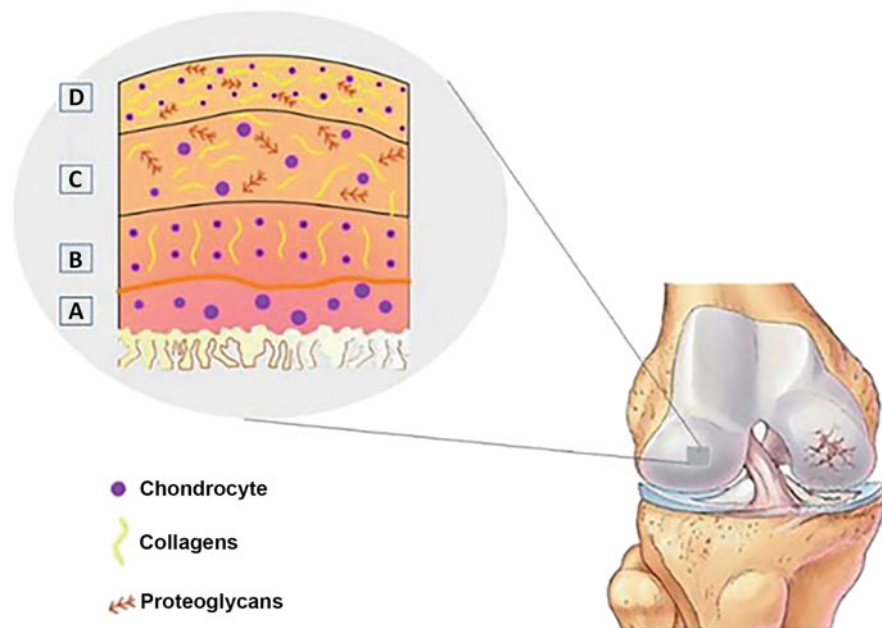


Fig. 11.3 The macro and microstructure of articular cartilage consist of four zones. (a) Calcified zone, (b) Deep zone, (c) Middle zone, and (d) Superficial zone. Obtained from reference (Farokhi et al. 2020) with permission

osteochondral allografting, and periosteal or perichondrial tissue grafting are several advanced methods developed to repair articular cartilage defects (Hangody and Füles 2003; Clair et al. 2009). Unfortunately, all of these techniques have certain disadvantages. The outcome after long-term follow-up is also unreliable due to degenerations such as fibrosis and calcification (Magnussen et al. 2008; Bhosale and Richardson 2008). The essential step to solve this issue is to repair tissue defects with regenerated tissue with hyaline cartilage characteristics (Dai et al. 2010).

Cartilage tissue engineering is an emerging method to treat AC injuries while restoring joint function in OA and other joint diseases (Dai et al. 2010). This method combines appropriate cells, biomaterial sources, and well-designed scaffolds. Chondrocyte cell lines, adult mesenchymal stem cells, and embryonic stem cells are appropriate cell sources used in cartilage tissue engineering. Fabrication of desirable scaffolds that can be well matched with native tissue in terms of mechanical properties and biocompatibility is also required in this method for successful cartilage tissue regeneration. Controlled biodegradability, the ability to form in different shapes and sizes to mimic the native features of the cartilage, porosity, and the ability to increase cell differentiation are the other design criteria that should be considered (Dai et al. 2010).

Hydrogels, fibers, microparticles, and 3D-printed scaffolds can be formed by self-material or by combining different materials to fabricate a hybrid composition and

improve its performance (Farokhi et al. 2020). A few cartilage tissue engineering-related products have made it to market using straightforward tissue-engineered techniques such as autologous chondrocytes. Scaffolds, growth factors, and gene delivery are a few examples of sophisticated tissue engineering techniques that have begun to advance to clinical trial phases (Keeney et al. 2011).

11.7 Using Shape-Memory Polymers in Cartilage Tissue Engineering Scaffolds

Scaffold implantation via traditional open surgery, especially in space-limited joints, leads to secondary psychological and physiological damage for patients (Xuan et al. 2020). The minimally invasive surgical approach provides a way to address the aforementioned issues, but it is not applicable for traditional scaffolds with predetermined sizes and shapes bigger than the arthroscopic channel. Recently, shape-memory polymers have been considered adaptable candidates to make suitable devices for minimally invasive implantation (Xuan et al. 2020). These devices can be fixed to a temporary compact state for convenient operation, and they will automatically restore their permanent predetermined shape after implantation. Despite their great advantages, shape-memory synthetic scaffolds are rarely studied in tissue engineering. Most are not practical for in-vivo applications due to the irrelatively high switch temperatures (Zhang et al. 2014; Baker et al. 2016). In particular, minimally invasive implantable shape-memory scaffolds for cartilage regeneration have not been studied.

Hydrogels are a scaffold alternative that can solve the drawbacks of other scaffolds used in cartilage tissue creation. Water or biological fluids can be absorbed and reabsorbed by hydrophilic polymer-based hydrogels having a three-dimensional structure without altering or degrading their chemical composition. Hydrogels are ideal biomaterials, they contain soft and rubbery components similar to the ECM of natural cartilage (He et al. 2021; Farokhi et al. 2020; Drury and Mooney 2003). The porous hydrogels can transfer low molecular weight solutes and nutrients, which is an important process for cell behavior (Delaey et al. 2020; He et al. 2021).

The specific properties of hydrogels are the minimally invasive approach for use in comparison to open surgery. It allows patients to have a shorter convalescence period and less pain. Moreover, it can easily fill irregular geometrical defects due to its injectability and in-situ formation (Drury and Mooney 2003; Solouk et al. 2014). The cross-linked structure of hydrogels provides an opportunity to maintain their shape and size into the aqueous phase and present proper mechanical features. There are several cross-linking methods to make hydrogels with stable structures. These biodegradable and biocompatible hydrogels might be prepared chemically and physically. Thus, various cross-linking techniques can be applied to extend the mechanical strength of hydrogels, including escalating the cross-linking density, adding the second phase, and improving chemical structure in the presence of physiological situations (Dai et al. 2010).

Because of their biocompatibility and capacity to replicate the ECM, hydrogels based on PEG, alginate, self-assembling peptides, hyaluronic acid (HA), or collagen have been widely explored in typical 3D scaffolds (Ramaraju et al. 2020; Solouk et al. 2014). Because implanting hydrogels often requires invasive surgical procedures such as arthrotomies (Funayama et al. 2008; Bédier et al. 2015), injectable hydrogels have been seen as an intriguing way to fill defects (Funayama et al. 2008; Bédier et al. 2015). Since they can give the capacity to twist within the needle, then instantly recover back to the original form of the defect, injectable scaffolds with shape-memory capabilities are required for retaining mechanical qualities and preventing leakage (He et al. 2021; Farokhi et al. 2020). It has been demonstrated that cryogels, a subtype of hydrogels created by crystallization at subfreezing temperatures, have shape-memory capabilities (He et al. 2021).

Many synthetic and natural biodegradable polymers have been used for articular cartilage tissue regeneration. Even though synthetic polymers such as PLA, PGA, and PLGA can easily be formed into designed shapes with relatively high mechanical strength, their hydrophobic surface is not desirable for cell seeding (Dai et al. 2010). Natural biomaterials with an intrinsic ability to mimic extracellular matrix and biocompatibility have attracted much attention (Dai et al. 2010).

Shape-memory materials, especially hydrogels, are considered a unique group of intelligent materials. High deformability and stimuli-responsive properties allow these materials to restore their original shape after exposure to an appropriate external stimulus, including temperature, pH, salt, specific (bio) chemical signals, and electric fields (cartilage). Hydrogels may also be cross-linked sequentially using protease degradable peptide cross-links and UV light-induced (Keeney et al. 2011). These differential network structures provide spatial control of cell fate in 3D structure by permitting or inhibiting cellular remodeling depending upon the cross-links. This system independently controls biochemical and biophysical properties of cell culturing microenvironments *in situ*. Combined with spatial controls, these techniques allow direct light patterning of physiologically functionalized cell-laden gels to imitate the diverse cartilage structures (Keeney et al. 2011). A study reported that in a hydrogel prepared through a double cross-linking process via Sodium Alginate and Gelatin, the first cross-linking agents were produced through a reaction between the aldehyde group in alginate and amino group in gelatin. In addition, UV light can form the second hydrogel network to control and improve the mechanical properties for cartilage tissue engineering (Farokhi et al. 2020). Chemical cross-linking can involve covalent bonds or add chemical components that produce a strong structure compared to the physical cross-linking methods. They present high mechanical properties and have a controlled degradation rate, which leads to a sustainable drug release. However, they are often toxic compounds (Mano et al. 2007; Shamekhi et al. 2017). Generally, enzymatic cross-linking, photo-crosslinking, and click reaction are considered chemical cross-linking strategies (Farokhi et al. 2020).

11.7.1 Alginate as a Smart Natural Polymer in Cartilage Tissue Engineering

Alginate is a natural biodegradable shape-memory polymer that exhibits appropriate physical features for cartilage tissue application. It is a naturally occurring anionic polysaccharide typically found in brown algae. Its hydrophilicity and high water absorbance capacity make it a suitable compound for cell growth. The extracellular matrix (ECM), which supports cell adhesion through chemical changes, has a structure comparable to this natural polymer (Farokhi et al. 2020). The fact that b-D-mannuronic acid (M) and a-L-guluronic acid (G) residues are connected in a copolymer (1–4) with variable ratios accounts for alginate's adjustable features. Alginate's weak mechanical strength can be improved using a physical and chemical cross-linking method.

Therefore, a covalent cross-linking method such as shape-memory alginate can be an appropriate method to overcome alginate's instability and let it precisely determine its shape (Tian et al. 2019). In spite of the fact that alginate is relative youth, shape-memory alginate is developing rapidly and provides an appropriate opportunity for that to prepare smart scaffolds which can be well matched with natural tissues in terms of biocompatibility and mechanical properties (Farokhi et al. 2020).

11.7.2 Alginate Scaffolds in Cartilage Tissue Engineering

Cartilage tissue engineering scaffolds based on alginate can be classified into injectable and preformed scaffolds. Injectable scaffolds such as hydrogels require the strong mechanical properties achieved by double networks, composite, and topological hydrogels for load-bearing applications. The poor mechanical strength of hydrogels corresponds to (1) the presence of high water in the structure, (2) lacking regular spreading of cross-linking per unit volume, (3) variety of polymer chain length, and (4) growth of crack due to energy accumulation (Costa and Mano 2015).

The calcium alginate hydrogels cannot maintain a certain shape and size when in contact with body fluids (Augst et al. 2006). The macroporous alginate hydrogel displays better physical and mechanical characteristics with shape memory. Its porosity can facilitate cell migration and drug and biomolecule agent delivery (Farokhi et al. 2020; Augst et al. 2006). In a study, different degrees of the covalent cross-link were used to produce the alginate hydrogel with the predefined shape. It is reported that the degree of cross-linking agents can define the recovery amount of the original shape. After that, the best cross-linking degree could be optimized, and the shape-memory alginate scaffold was utilized for cell delivery and growth factor (Farokhi et al. 2020; Augst et al. 2006). In another study, an aligned pore structure was prepared with the combination of shape-memory alginate, collagen type I, and collagen type II for cartilage regeneration. They have found that the shape-memory

properties of scaffolds can be used in minimally invasive surgeries and cell delivery (Bencherif et al. 2012).

11.7.3 Alginate as a Cell Carrier in Cartilage Tissue Engineering

Stem cells and chondrocytes are used mainly in cartilage tissue engineering (Moreira-Teixeira et al. 2011). In addition to the potency of stem cells in self-renewal and differentiation in tissue engineering, their capacity to produce signaling chemicals per the cell microenvironment is crucial for regenerating new tissue (Makris et al. 2015). Based on other studies, there are three promising types of stem cells for repairing cartilage damages: (1) multipotent such as mesenchymal stem cells (MSC), (2) pluripotent such as embryonic stem cells (ESC), and (3) induced pluripotent stem cells (iPSC) (Lam et al. 2017). Alginate microparticles are employed for delivering cells through alginate hydrogels. In this case, after mixing the cell suspension and sodium alginate, the solutions were drawn in a 21-gauge syringe to incorporate cells in alginate microcapsules. The alginate microcapsules containing cells were then dipped in a CaCl_2 solution to obtain alginate hydrogel (Farokhi et al. 2020).

Chondrocytes used in cartilage tissue engineering could be isolated from cartilaginous donor organs like the trachea, nose, and knee. Using autologous chondrocytes had some drawbacks. For example, the lack of chondrocytes population results in in-vitro expansion, which leads to a fibroblast-like phenotype change. Moreover, harvesting healthy chondrocytes from undamaged cartilage requires surgery (Moreira-Teixeira et al. 2011). Using stem cells or co-culture of stem cells and chondrocytes has been increased to overcome these limitations (Moreira-Teixeira et al. 2011; Lam et al. 2017). Despite the drawbacks of chondrocytes, many studies reported that chondrocyte delivery via alginate hydrogels is capable of cartilage tissue engineering (Mirmusavi et al. 2022). Selecting an appropriate cell source that can mimic natural chondrocyte ECM is one of the substantial points in cartilage tissue engineering (Jakobsen et al. 2010). Collagen type II and Aggrecan are the major components of cartilage ECM that analyze for chondrogenesis (Mauck and Burdick 2011). Chondrocytes and ADSCs encapsulated in alginate microparticles were studied for cartilage tissue engineering. Chondrogenic media with and without TGF β 3 were consumed in both cell cultures for 3 weeks. Until day 14, the differentiated cells from ADSCs expressed more aggrecan in both media compared to chondrocytes, but on day 21, the cells in the medium without TGF β 3 synthesized more aggrecan. This trend also occurred for chondrocytes.

The researcher suggests that ADSCs may be a hopeful source for cartilage tissue engineering (Ansar et al. 2012). Chondrocytes can be harvested from various tissues, including articular cartilage, nasal cartilage, or ear cartilage (Pleumeekers et al. 2014). Researchers isolated autologous nasal chondrocytes from rabbits and expanded them in monolayer and 3D alginate cultures. The in-vitro results showed that the proliferation rate in 3D culture was faster, and chondrogenic markers were up-regulated. In-vivo results demonstrated the formation of hyaline-like tissue with

matching mechanical properties with natural cartilage. They concluded that the delivery of nasal chondrocytes by means of alginate hydrogel might be an appropriate choice for cartilage tissue engineering (Chen et al. 2018). The effect of chlorogenic acid, a plant extract, on encapsulated chondrocytes in alginate was investigated in vitro and in vivo. In-vitro analyses revealed that chondrogenesis increases in the presence of chlorogenic acid. In-vivo injury models in chicks also proved the results because more chondrocyte proliferation and ECM secretion were observed. The study suggests that combining chlorogenic acid, alginate, and chondrocyte can improve defected cartilage healing (Cheng et al. 2018).

Delivery of biomaterials to defected zones in a less invasive manner is a new trend in biomedical engineering that can be fulfilled via the injectable hydrogel. Solidification occurs inside the body due to changing pH, temperature, or the presence of ions such as Ca^{2+} (Farokhi et al. 2020). Since alginate cross-linking bonds are based on ionic interactions, injectable hydrogels could be an attractive polymer. Researchers investigated the encapsulation of chondrocytes in the alginate-gelatin self-cross-linkable injectable hydrogel. They studied viability, proliferation, migration, and aggrecan and collagen expression. The results showed that the produced injectable hydrogel could be suitable for cartilage tissue engineering (Cheng et al. 2018). Researchers investigated the use of human dental pulp stem cells, extracted from the perivascular niche of the dental pulp (hDPSCs) and cultured in 3% alginate hydrogel, for rabbit cartilage regeneration. The results showed that hDPSCs in a chondrogenic medium express collagen type II and aggrecan. Cartilage regeneration was also observed 3 months after surgery (Mata et al. 2017). Another research group developed a hyaluronic acid-alginate hydrogel microsphere for delivering stem cells. The periodontal Ligament Stem Cells (PDLSCs) were encapsulated in HA-alginate hydrogels and investigated regarding the chondrogenic gene expression and histological results. They stated that this system with cartilage gene expression (col II, SOX9, and aggrecan) and cartilage-like tissue regeneration could be an appropriate candidate for tissue engineering (Ansari et al. 2017).

Scaffolds utilized in cartilage tissue engineering must have unique characteristics, including controllable biodegradability depending on the tissue formation rate, biocompatibility, cell adherence, proliferation, and differentiation, as well as appropriate mechanical and physical qualities, and penetration capacity through interconnected pores. A 3D polymer scaffold should provide a structure matched with the biomechanical structural properties of the native ECM. Therefore, selecting appropriate materials to fabricate desirable scaffolds with these features is critically important (Glicklis et al. 2000). Due to their biocompatibility and low cost, natural polymers are suitable materials for cartilage tissue engineering applications, easy handling, and good cell interaction. Among those, alginate is a suitable material for fabricating scaffolds, using proteins, peptides, and inorganic and organic biomaterials to modify its structure. Alginate matrices have been widely used in medicine, especially cartilage tissue (Farokhi et al. 2020). In a study, a research group produced a three-dimensional spongy alginate scaffold to support chondrocyte culture since the pores can provide suitable spaces for cell migration [Ref].

Moreover, they evaluated the effect of hyaluronate during the production of chondrocytes (Miralles et al. 2001). Another study established a new method to generate alginate-based foams to improve their flexibility, pore structure, and mechanical properties compared to the other techniques. Researchers attempted to decrease the water in the structure using the drying technique. They also investigated the impact of the alginate's molecular weight on the foams' tensile strength (Andersen et al. 2012). The alginate-based composite gel scaffolds, including hydroxyapatite and gelatin microspheres, have been fabricated in a study. Hydroxyapatite and gelatin microspheres can improve mechanical strength, swelling ratio, gelation time, and bioactive performance (Yan et al. 2016).

11.7.4 Alginate in the Electrospinning Process

Because they imitate the natural ECM, nano and microfibers have been employed extensively in tissue engineering applications. The method is based on applying a high voltage to a polymer solution which can be stretched as fibers from the syringe needle toward a collector. Polymer solidification occurs during the process due to solvent evaporation. Nano and microfibrinous mats provide a high specific surface area which can positively affect cell response (Bonino et al. 2011; Tamayol et al. 2013). The nanofibers' physical, chemical, mechanical, and biological characteristics can be tuned by adjusting some properties, such as viscosity or concentration of the solution, flow rate, and electrical power (Ji et al. 2016). Biomass biocompatibility can be affected by fiber diameters, porosity, and alignment (Jeong et al. 2010). As mentioned, an ideal scaffold should have a structure similar to the native ECM. The Nano fibrous alginate matrix is comparable to the fibrous protein of ECM. However, fabricating alginate nanofibers is a challenge since the gelation of the alginate solution starts to occur at lower alginate concentrations (Venkatesan et al. 2015). Therefore, the viscosity is insufficient to form the fibers, leading to the generation of short fibers containing the beads. On the other hand, it is very hard to inject a solution with high viscosity (Farokhi et al. 2020).

11.7.5 Alginate in a Multiresponsive System

Alginate intrinsically has the capacity to respond to the pH of the environment. Therefore, its combination with a thermoresponsive material provides an opportunity to produce a dual-stimuli-responsive alginate hydrogel. These beneficial vehicles can participate in locally released drugs or biomolecule agents in response to a certain pH and temperature (Farokhi et al. 2020). A research group has fabricated a thermo- and pH-sensitive alginate hydrogel whose surface was grafted with various mass ratios of N-isopropyl acrylamide (NIPAAm). In that study, the effects of pH and temperature on the swelling and deswelling rate were evaluated. It was recorded that a suitable mechanical strength during the swelling period was achieved while the sensitivity of hydrogel to pH and temperature was enhanced

(Zhang et al. 2005). In another study, a semi-sensitive hybrid hydrogel composed of bacterial cellulose nanofibers and sodium alginate was evaluated. The Bacterial cellulose nanofibers and sodium alginate are sensitive to electrical and pH stimuli. The hybrid hydrogels presented better-swelling properties than pure sodium alginate hydrogels (Shi et al. 2014). Polyethylene oxide (PEO) was studied as a second phase to generate Alginate-PEO. PEO could reduce the viscosity of the alginate solution to enhance its spinnability in the electrospinning process (Bhattarai et al. 2006). It should be considered that the alginate fibers have a low mechanical strength which can be reinforced by adding inorganic materials such as carbon nanotubes (CNTs). A uniform distribution of CNTs in an alginate matrix at an optimal concentration can improve the mechanical strength of the matrix (He et al. 2012). Alginate-based chitosan fibrous scaffolds were studied for cartilage repairs. Alginate-based chitosan fibers have enhanced the attachment and proliferation of chondrocytes compared to pure alginate fibers. They are also the best choice for cartilage regeneration due to their suitable physicochemical and biological properties (Iwasaki et al. 2004).

11.8 More Studies and Reviews

Microtia is a deformity of the outer ear. It is a type of birth defect and happens when the auricle does not fully form during the first weeks of pregnancy. The most common treatment to reconstruct the auricle is using the patient's costal cartilage carved into an auricular frame (Uto et al. 2020). Typically, patients don't have costal cartilage restoration until they are 10 years old. Less intrusive techniques must be developed since the thorax is the donor location for costal cartilage harvesting, which results in high donor-site morbidity (Uto et al. 2020).

Cartilage regenerative medicine is a possible approach to reducing the invasiveness of therapies for cartilage abnormalities, particularly microtia. Numerous studies have documented the creation of three-dimensional regeneration of cartilage using scaffolds and cells with high mechanical strength and the right form. Researchers have developed a novel technique for creating cartilaginous particles from human iPS cells. Using this technique, a new scaffold with three-dimensional shape-memory properties was made to produce regenerating cartilage in the shape of an auricle. It was the form of auricular frames in three dimensions, made up of a helix and an antihelix. It was made to seem like an auricular framework that was carved from a patient's costal cartilage (Uto et al. 2020). The existing method, nevertheless, has several drawbacks. For instance, only a certain amount of cells may be extracted from the harvested auricle. More cells are needed for auricular reconstruction than for nose dorsum augmentation, which makes the donor site more invasive. In addition, cartilage grows after implantation, similar to many other documented techniques, making the success of this treatment questionable (Uto et al. 2020). A study team described a method for regenerating the auricles using cartilaginous particles derived from two separate lines of human iPS cells (Uto et al. 2020). They claimed that PLA and its copolymers exhibit a negligible degree of thermoresponsive shape memory. When materials are heated to a temperature

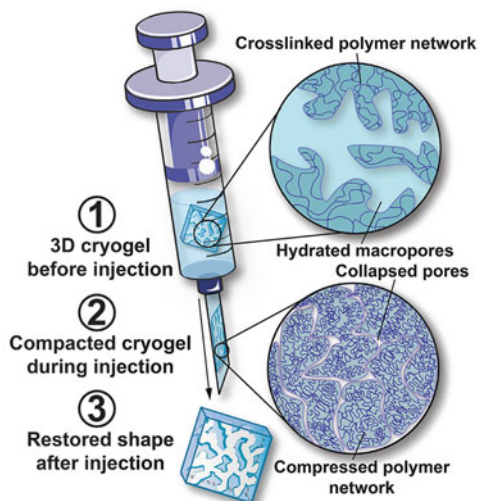
above their glass transition or melting temperature, then cooled to a temperature below the glass transition temperature, the thermoresponsive shape-memory effect occurs. Around 60 °C is the glass transition temperature for PLA. This suggested that PLA could be capable of thermoresponsive form memory at 121 °C for autoclave sterilization. In combination, heating and chilling may be used to create a unique scaffold with biocompatibility, the capacity to hold onto cartilaginous particles and promote their survival and development, and shape memory (Uto et al. 2020).

Shape-memory cryogels made of hyaluronic acid offer an excellent microenvironment for chondrocyte adhesion, proliferating, and matrix secretion to heal cartilage damage. Cryogels may be created utilizing organically produced polymers, lowering the potential for toxicity. Cryogels' sponge-like elastic qualities allow them to regain their original shape after injection while having no adverse effects on the metabolism or survival of the encased cells. Using a single, minimally invasive injection of a cell-encapsulating biocompatible 3D scaffold that can reconstitute itself to suit the defect geometrically and facilitate matrix regeneration, they provide a clinical potential to fill focal cartilage lesions (He et al. 2021). Experts created many macroporous biomaterials with shape-memory capabilities to create a favorable environment for chondrocyte adhesion, proliferation, and matrix formation (He et al. 2021). For comparison with traditional HA-based hydrogels, they created HA-based cryogel scaffolds with and without RGD, a cell adhesion peptide, to assess how cells interact with the material and examine its mechanical characteristics (He et al. 2021). Then, they implanted primary chondrocytes within the gels to give the cells a microenvironment suitable for in-vitro cultivation. After being injected with a tiny pore needle, they said chondrocytes infused into HA-based cryogels would still be metabolically active. (ii) When compared to mesoporous HA-based hydrogels, the macroporous and highly interconnected structure of cryogels would offer an unrestricted environment for cell development and diffusion of oxygen and nutrients, enhancing chondrocyte metabolism and matrix synthesis (He et al. 2021). Figure 11.4 shows an example of shape-memory cryogel injection in cartilage defects (He et al. 2021).

11.9 Conclusion

The application of shape-memory polymers in the biomedical sector is very promising. Shape-memory composites have exploded in popularity in biomedical research during the last few decades. Because of their capacity to memorize complicated configurations that differ from their constant shapes, shape-memory polymers are ideal candidates for constructing tissue-engineered constructs. As a result of their modest temporary shape that can grow to their ultimate (patient-specific) shape following in-vivo implantation, they enable minimally invasive surgery. Additionally, they make it possible for complicated mechanical deformation to be performed automatically rather than requiring manual manipulation by a surgeon. One of the earliest publications on shape-memory polymers was a 1941 patent for an elastic

Fig. 11.4 Injection of shape-memory cryogels into defect created in vitro. Obtained from reference (Eggermont et al. 2020) with permission



polymer resin with “elastic memory” for dental uses, demonstrating the material’s potential. This patent states the vinyl acetate, (m)-ethyl methacrylate, vinyl chloride, and copolymers-based resin. In recent years, shape-memory polymers have drawn much attention and exploration for their potential use in biomedicine.

Two significant obstacles to cartilage tissue regeneration are cartilage tissue’s poor reparative and regenerative ability due to its avascular nature and challenging scaffold insertion by typical open surgery due to space-restricted joints. Bioactive body temperature-responsive shape-memory scaffolds are used to solve these problems concurrently. These scaffolds are easily implantable using a minimally invasive technique, and the patient’s body temperature will help them heal. As a core component of tissue engineering, polymer scaffolds have a crucial influence on cartilage regeneration due to their pivotal role in ensuring biocompatibility and mechanical strength. Many synthesized and natural biodegradable polymers have been utilized for articular cartilage tissue regeneration. Shape-memory scaffolds form hydrogels, fibers, microparticles, and 3D-printed scaffolds to improve their performance.

Shape-memory materials, especially hydrogels, can be considered mechanically active or smart materials. They can restore their original shape in response to an appropriate external stimulus, including temperature, pH, salt, specific (bio) chemical signals, and electric fields. The minimally invasive approach is regarded as one of the specific properties of hydrogels compared to open surgery. It provides an opportunity for patients to have less pain and a shorter convalescence period. Its injectability and in-situ forming can easily fill irregular geometrical defects. PEG, alginate, self-assembling peptides, hyaluronic acid (HA), due to their biocompatible qualities and capacity to simulate the extracellular matrix, and collagen, have received substantial research as typical 3D scaffolds.

Acknowledgments Part of the research reported in this paper was supported by National Institute of Dental & Craniofacial Research of the National Institutes of Health under award number R15DE027533, 1R56 DE029191-01A1, and 3R15DE027533-01A1W1.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Widely Used Biomaterials in Cartilage Biofabrication

12

Farzaneh Jabbari, Babak Akbari, and Lobat Tayebi

Abstract

Cartilage, a nonvascular type of supporting connective tissue, is a flexible tissue with a less organized microarchitecture than bone. Perichondrium separates it from surrounding tissues and consists of an outer fibrous layer and an inner cellular layer. There are three types of cartilage with different structures and functions: hyaline cartilage, the most common type of cartilage with a smooth surface and many collagen fibrils and chondrocytes, fibrocartilage, the toughest type of cartilage due to dense bundles of collagen fibers embedded with chondrocytes, and elastic cartilage, which is flexible and resilient due to existence of elastic fibers. This tissue is avascular, and cells receive nourishment through diffusion from the surrounding microenvironment. The avascular nature is the main reason for its inability to repair. Many pathologies can affect this tissue, such as traumatic rupture, osteoarthritis, and osteochondritis dissecans, which result from inflammatory and degenerative causes. Many biological strategies for cartilage regeneration are challenging due to the low intrinsic capacity of this tissue for repair. However, innovative materials and techniques can significantly accelerate cartilage regeneration with various merits and drawbacks. Many natural and synthetic biomaterials, various growth factors, and many types of cells are

F. Jabbari

Nanotechnology and Advanced Materials Department, Materials and Energy Research Center (MERC), Tehran, Iran

B. Akbari (✉)

Life Science Engineering Department, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran

e-mail: babakbari@ut.ac.ir

L. Tayebi

Marquette University School of Dentistry, Milwaukee, WI, USA

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M. Baghaban Eslaminejad, S. Hosseini (eds.), *Cartilage: From Biology to Biofabrication*, https://doi.org/10.1007/978-981-99-2452-3_12

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used to improve cartilage regeneration. Stem cells, especially mesenchymal stem cells, are considered promising for cartilage repair in combination with other active biological factors. Biomolecules mediate cell attachment, migration, proliferation, and senescence. Growth factors improve cells' chondrogenic differentiation, and tissue integration is promoted. One of the most attractive smart materials is piezoelectric, which is used as electroactive scaffolds for tissue engineering. These materials transfer electrical stimuli directly into the body, and this power regulates repairing pathways and increases the regeneration rate. Piezoelectric scaffolds remarkably improve cell functionality without adding growth factors or other bioactive molecules. However, they are toxic or nondegradable and must be modified or combined with biodegradable polymers for clinical application. The present chapter gives important and primary insight into cartilage structure, diseases, and frequently used materials for its regeneration and introduces novel materials useful for cartilage repair. Essential and basic information about biomolecules, piezoelectric materials, and their advantages and limitations are presented. It can be concluded that, despite profound advances in cartilage tissue engineering, there are still severe challenges with biomaterials used for cartilage repair that must be overcome to introduce a definitive treatment method.

Keyword

Biomaterials · Piezoelectric materials · Cartilage regeneration · Stem cells · Growth factors

12.1 Introduction

Biofabrication is the automated production of tissues and organs to address health challenges in medicine. It is a fantastic tool for tissue engineering and regenerative medicine applications, which has an incredible ability to produce implantable 3D structures containing biomaterials and living cells, introduced as bioscaffolds. The important point in this regard is that these functionalities in the clinic are facing a severe challenge. For example, scaffold-based tissue regeneration techniques are applied to produce macroscale features, including scaffolds fabricated by solvent casting, electrospinning, freeze-drying, gas foaming, and other widely used methods. Live cell distribution in these methods during fabrication is so poor. It needs cell seeding after the fabrication, leading to highly homogeneous structures that do not mimic the body's natural tissues. Many factors such as cell-cell interactions, signaling molecules, substrate adhesion, and mechanical forces determine the cellular arrangements in natural tissues. These interdependent factors improve extracellular matrix formation, accelerating damaged tissue repair (Harley et al. 2021). In recent years, various biomaterials have been used in various ways to regenerate cartilage tissue. To know all the techniques of cartilage tissue regeneration, it is necessary to study the biological structure of this tissue. Cartilage is an

essential structural component of the body. It is a firm tissue, but it is softer and much more flexible than bone. This tissue comprises “chondrocytes,” or specialized cartilage cells, which are responsible for synthesizing collagen and proteoglycan. Chondrocytes’ diameters vary between 7 and 30 μm according to the anatomical layer and can synthesize extracellular matrix (ECM) consisting of collagen type II, IX, XI, and proteoglycans (Akkiraju and Nohe 2015). Cartilage, a connective tissue, is found in the ends of the ribs, ears, nose, bronchial tubes, or airways, between the vertebrae in the spine, and joints between bones. The primary duties of cartilage are providing an appropriate framework for bone deposition, covering the surfaces of joints and reducing friction between them, and acting as a shock absorber. There are three types of cartilage: hyaline cartilage, elastic cartilage, and fibrocartilage. Hyaline cartilage is the most common cartilage found in the ribs, nose, larynx, and trachea; it organizes the embryonic skeleton.

Elastic cartilage, or in other words, yellow cartilage, is found in the external ear, epiglottis, and larynx, and elastic bundles are observed in this tissue which makes it stiff, yet elastic. Fibrocartilage or white cartilage is found in intervertebral discs, joint capsules, and ligaments and provides excellent tensile strength for tissue. It is usually a transitional layer between hyaline cartilage and tendon or ligament and consequently does not have a perichondrium. Cartilage is responsible for transferring and distributing load when the joints are moving. When joints are stimulated, cartilage acts like the layers that carry the weight of the stimulus: either transmitting or dividing it. In other words, the cartilage between the bones plays the role of ball bearings and shock absorbers and is an integral part of our movements. Cartilage is very similar to bone tissue, but unlike bone, calcium deposition does not occur in it, and therefore this tissue is very elastic (Grässel and Aszódi 2016).

12.2 Properties of Cartilage Tissues

Cartilage has a porous matrix of collagen fibers and a fluid rich in proteoglycans, creating osmotic pressure in the tissue. In addition, hyaluronic acid presents excellent protective and antifrictional properties. Low friction at joints can cause smooth joint motion, and the roughness of the articular cartilage exacerbates this issue. Cartilage composition and its components’ spatial configuration have many effects on its mechanical and physiological properties (Perni and Prokopovich 2020). Many researchers have shown that, at low stress, articular cartilage is significantly more viscous and therefore does not present the physical behavior under a physiological stress range. Articular cartilage at a high-induced stress range shows more elastic behavior than the walking stress range. Off-bone cartilage shows a frequency-dependent loss modulus, causing enormous energy dissipation, while on-bone articular cartilage is frequency-independent (Lawless et al. 2017). Articular cartilage is a heterogeneous tissue in compression and tension, presenting anisotropic and nonlinear behaviors and depth-dependent relaxation behavior under unconfined compression. Its thickness is 2–4 mm, and unlike many tissues, it lacks blood vessels, nerves, and lymphatic tissue (Lawless et al. 2017).

12.3 Cartilage Diseases

Osteochondritis dissecans (OCD) occurs in the joints because the lack of blood in the joint causes the bone inside to soften. Subsequently, a small piece of the bone dies and separates from the larger bone. This small bone and the covering cartilage crack and break loose and move into the joint area, making it unstable. The etiology of this lesion is not known. However, many studies showed that abnormal vascular anatomy, abnormal ossification of the epiphysis, trauma, endocrine imbalances, or combination could cause OCD. A magnetic resonance imaging scan (MRI) is an effective tool to detect OCD, which appears as crescent-shaped defects in the bone. In children and younger teens with stable lesions, nonsurgical methods are often used for treatment, while surgical management is the best treatment for unstable lesions. There are three surgical techniques: (a) creating a path for new blood vessel formation by drilling a hole into the damaged tissues, (b) fixing the damaged tissue in place with screws, (c) inducing new tissue formation in the damaged area by using graft (Krishnan and Grodzinsky 2018). Relapsing polychondritis (RP) is a systemic inflammatory disease of unknown etiology that can be dangerous. The disease affects cartilage tissue in the ears, nose, throat, and lungs. The cause of this disease is not yet known, but it is probably an autoimmune disease. The signs and symptoms of RP are different in many people and depend on the parts of the body that are affected. Some of the most important symptoms of this disease are hearing loss, ear infections, balance disturbances, joint pains and swelling, blood vessel inflammation of the large arteries, shortness of breath, and stridor (high-pitched sound during breathing). The diagnosis of RP often occurs late since its many symptoms are similar to and mistaken for other, more common, diseases. Biopsy of inflamed tissues is frequently used to diagnose RP. A biopsy is a medical procedure that involves taking a small piece of tissue and examining it under a microscope. A blood test alone is not enough to diagnose RP; lung function tests and computed tomography (CT) scan pictures effectively determine the severity of the disease. Despite extensive research about this disease, an effective and definitive treatment method has not been determined. Treatment depends on which organs in the body are affected and how seriously. RP can be controlled with medication such as methotrexate and steroids. Nonsteroidal anti-inflammatory drugs are useful when the skin and joints are involved, but for more severe RP, immunosuppressive drugs are used. The long-term outlook for RP is so different from person to person. In recent years, patients with severe cases of RP continue to live long and fulfilling lives, and life expectancy among patients has increased with the introduction of modern medicine and new treatment techniques (Krishnan and Grodzinsky 2018). Chondrocalcinosis or cartilage calcification, also known as calcium pyrophosphate deposition (CPPD), is another serious disease that threatens cartilage tissue. The main symptoms of this disease are osteoarthritis, pseudogout arthritis, and symptoms similar to rheumatoid arthritis. Injuries such as trauma that destroy collagen and other cartilage protein structures due to the lack of magnesium or excess calcium or iron, cause calcium crystal deposition. CPPD's most common treatment techniques are intra-articular glucocorticoid injections and nonsteroidal anti-inflammatory drugs (NSAIDs)

(Kumar et al. 2017; Wang et al. 2019). Cartilage tumors are very dangerous and are of two types, benign and malignant tumors. Enchondroma and osteochondroma are the most common benign cartilaginous tumors, and chondrosarcomas are malignant tumors. Chondrosarcoma is a bone sarcoma that develops in the cartilage cells. Chondrosarcoma significantly affects the thighbone, shoulder, or pelvis cartilage cells. Less often, it starts in the knee, ribs, skull, and windpipe. Symptoms of chondrosarcoma depend on where the tumor is and how big it is. There are several stages for this: (1) the cancer is low-grade and has not grown outside the bone, (2) the cancer is high-grade and has spread beyond the bone, (3) spreading cancer to another part of the body. Surgery is the first and most common treatment for this disease. The tumor and parts of the surrounding natural tissue must be removed. The goal of this procedure is to preserve the organ where the tumor is located instead of amputating that organ. Another effective treatment is limb-sparing surgery; infected bone is removed and replaced with bone grafts in this technique. Sometimes cancer spreads from bone tissue to other parts of the body; in this case, the infected limb must be amputated. Sometimes the tumor is in a place that cannot be removed surgically. Therefore, chemotherapy can reduce the risk of cancer. When the tumor is in the spine or the skull, it is impossible to remove it; in this situation, a combination of chemotherapy, surgery, and radiation treatments is useful (Lu et al. 2020). Enchondroma is the most common type of hand tumor that begins in cartilage. The cause of this disease has not yet been determined. However, the following factors can be among the possible causes: (1) overgrowth of the cartilage that lines the ends of the bones and (2) persistent growth of original cartilage. The most common symptoms of this disease are severe hand pain when the tumor is large and slow bone growth in the affected area. It is frequently used to diagnose X-rays, radionuclide bone scans, MRI, and CT scans. Surgery and bone graft are common treatment methods. Osteochondroma is another common benign bone tumor, which a leftover piece of the growth plate that grows in the wrong direction causes it. The most important symptoms of this disease are numbness and tingling, color change in a limb due to vascular compression, and loss of pulse in the affected limb. Symptomatic osteochondroma needs to be removed by surgically; in some cases, the bone is broken to guide it to grow in the right direction (Krishnan and Grodzinsky 2018). Osteoarthritis (OA) is arthritis that affects the joints, usually in the hands, knees, hips, neck, and lower back. This occurs when the protective cartilage tissue of the bone wears out and disappears over time. There is no cure for OA, treatments are used to reduce inflammation and pain. Regular exercise, weight loss, and medications significantly improve strength, flexibility, and balance. Magnet therapy is one of the safe and uncomplicated treatment methods used in physiotherapy that relieves pain and accelerates the healing process of damaged tissue. Many clinical studies show that high-intensity laser therapy as a noninvasive treatment option is useful for decreasing pain in patients with OA. This method is new, and researchers do not know if it has any long-term risks; many in vitro studies have investigated short-term effects. Therefore, it is possible that long-term use of laser therapy can cause serious and incurable tissue damage, which will be revealed in the future with

further clinical studies (Menon and Mishra 2018; Gato-Calvo et al. 2019; Youssef et al. 2016).

12.4 Cartilage Tissue Regeneration Approaches

Tissue engineering uses engineering technology and biological knowledge to repair damaged tissues, cells, and organs. Many factors, including nanotechnology, biomaterials, drug delivery, and stem cells, play essential roles. Therapeutic approaches for regenerating damaged cartilage are categorized into three main groups, (1) symptomatic treatments, (2) clinically available treatments, and (3) under development methods. In Fig. 12.1, all the approaches for damaged cartilage repairing are presented (Medvedeva et al. 2018).

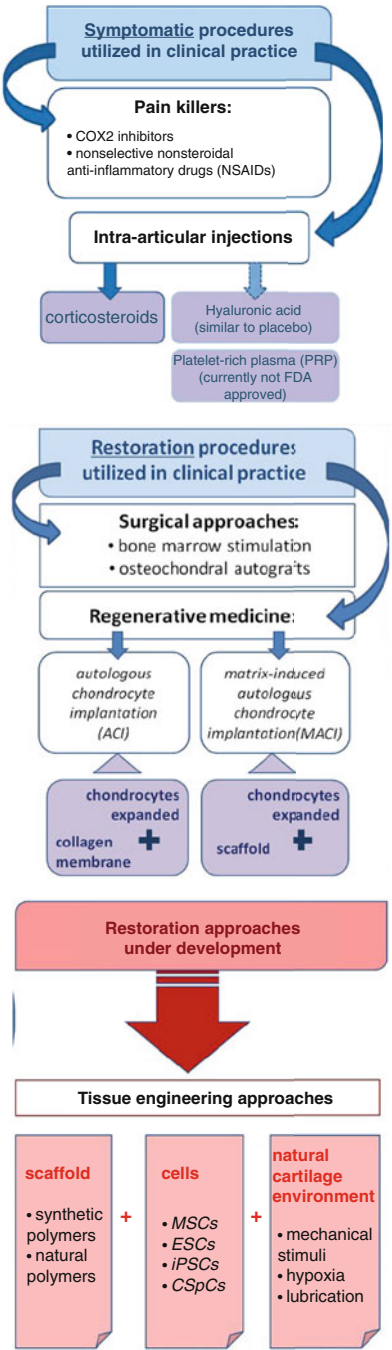
12.4.1 Clinically Used Approaches

Intra-articular injections of various compounds: this method is widely used for delivering specific compounds to the lesion area. Many studies recommend intra-articular corticosteroid injections as a fantastic tool for OA treatment and have observed satisfactory results. This method has the highest efficiency in the lowest dose, reducing the disease's progression. Another effective method for OA treatment is hyaluronan injection since OA significantly reduces the concentration of hyaluronan in synovial fluid and its molecular weight. Injection of Platelet-Rich Plasma (PRP) can significantly prevent the progression of OA, but its mechanism of action is still unknown. The FDA does not approve this method due to the lack of clinically satisfactory results (Shahid and Kundra 2017; Estades-Rubio et al. 2017; Jüni et al. 2015).

12.4.2 Surgical Approaches

Many researchers show that microfracture and similar methods are smart techniques for small defects treatment. This method creates narrow and small holes in the bone; access to the bone marrow is provided by penetrating the subchondral bone. Multipotent marrow stromal cells migrate to the defect site and accelerate new tissue formation. Due to its simplicity and low cost, this technique is frequently used (Lamplot et al. 2018). Replacing damaged cartilage with tissue grafts (osteochondral allograft and autograft) is another fantastic surgical method for tissue repair, in which a small cylindrical piece is placed in the lesion area. Its efficiency depends on various factors, including age, size of the lesion, and sex. The main drawbacks of this method are donor site morbidity, the immunologic response by the recipient, and the risk of disease transmission from the donor (Gracitelli et al. 2015).

Fig. 12.1 Schematic illustration of approaches for repairing damaged cartilage tissue. Abbreviations: ACI (autologous chondrocyte implantation), MACI (matrix-induced autologous chondrocyte implantation), MSCs (mesenchymal stem cells), ESCs (embryonic stem cells), iPSCs (induced pluripotent stem cells), CSPCs (chondrogenic stem/progenitor cells) (Medvedeva et al. 2018). (Licensed under an open access Creative Commons CC BY 4.0 license)



12.4.3 Regenerative Medicine and Cell-Based Approaches

The first methods to repair damaged cartilage tissue were very laborious and did not provide satisfactory clinical results. Amazing approaches are being taken to repair cartilage tissue with the advent of smart biomaterials and new treatments. Today, the most common therapies used in cartilage tissue engineering are (a) mosaicplasty: transplantation of small and cylindrical pieces of cartilage and bone to lesion area to provide an even resurfaced area, (b) microfracture: creating small holes in the surface of joints, which accelerate regeneration process, (c) autologous chondrocyte implantation: this is a procedure in which the chondrocytes are collected and cultured in the laboratory to increase cell number and then injected to the lesion area with a unique surgical technique to induce new tissue formation, (d) matrix-induced autologous chondrocyte implantation: in this innovative surgical technique, a patient's cells are used to repair cartilage defects in the knee, the damaged tissue is removed from the lesion area and transferred to the laboratory, after repairing, it is placed in the desired area again. In Fig. 12.2, all the clinically approved approaches to cartilage tissue regeneration are presented (Medvedeva et al. 2018). In all these techniques, applying some important points will increase the efficiency of the tissue healing process, for example, the addition of BMP-2 to the culture medium, 3D cultures, and decreasing the number of cell passages (Medvedeva et al. 2018). Cell-based approaches use mesenchymal stem cells (MSCs) from sources such as synovial membrane, bone marrow, adipose tissue, cord blood, and dental pulp tissue. The ability to differentiate into chondrocytes is very different for each cell. In using cell-based methods, the formation of fibrous tissue is possible, which can disrupt the formation of the main tissue. Therefore, it is necessary to follow all the principles of cell culture and differentiation to reduce side effects (Hulme et al. 2021).

12.4.4 Tissue Engineering Approaches

Scaffolds are one of the most important components of this method, which include a wide range of materials and are made with various techniques. Natural and synthetic polymers are widely used for cartilage tissue engineering. Natural polymers are biodegradable and biocompatible, but their compositions differ from batch to batch. There are various methods for fabrication of the scaffolds, such as molding, 3D bioprinting, electrospinning, freeze-drying, etc. Each of these methods has its benefits and drawbacks. In recent years, various commercially available tissue-engineered products of natural and synthetic polymers presented favorable clinical outcomes. Combining several approaches, including intelligent and advanced scaffolds, excellent differentiated chondrocytes, 3D printing of engineered constructs, and eliminating side effects can significantly enhance the regeneration of damaged tissue (Medvedeva et al. 2018).

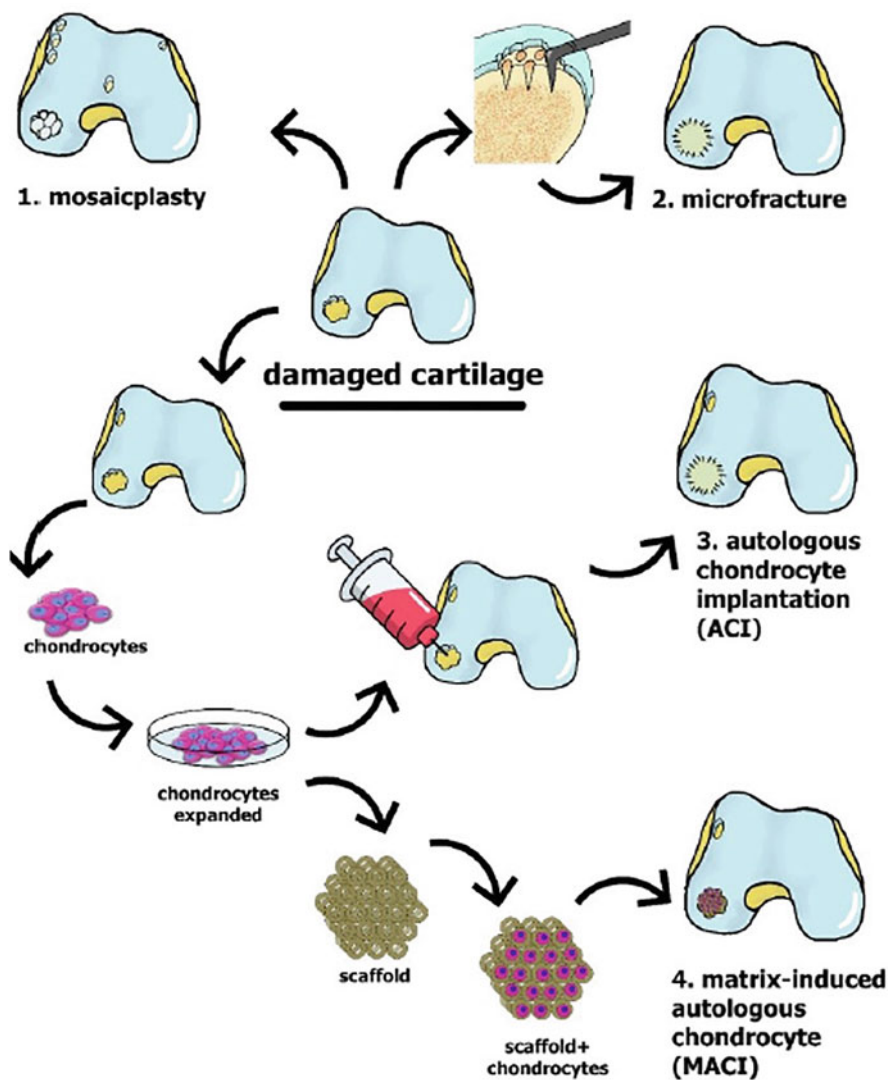


Fig. 12.2 Schematic representation of all clinically approved approaches to regeneration of cartilage tissue (Medvedeva et al. 2018). (Licensed under an open access Creative Commons CC BY 4.0 license)

12.5 Biomaterials for Damaged Cartilage Tissue Regeneration

Natural and synthetic substances are two main biomaterials used extensively for cartilage tissue repair. Of course, various modification techniques have been used for developing short-term and long-term structures for enhancing cell behaviors and the new tissue formation process.

12.5.1 Natural Materials

These biomaterials are used extensively for fabricating cartilage engineering scaffolds; this family's most important members are collagen, hyaluronic acid (HA), fibrin, chitosan (CS), agarose, alginate, cellulose, and chondroitin sulfate. These materials have many advantages: excellent biocompatibility, nonimmunogenic and nontoxic, biodegradability, excellent interaction with cells and tissues, high ability to support cell adhesion/migration/proliferation/differentiation, and low cost. The main disadvantages include disease transmission, possible immune reaction, low mechanical strength, and production process problems (Fadilah et al. 2022).

12.5.1.1 Collagen

Collagen-based biomaterials are ideal candidates for cartilage tissue regeneration, and collagen type II is the main component of articular cartilage and shows better chondrogenic ability than collagen type I. Therefore, collagen type II is considered the substrate of biomaterials for cartilage regeneration. It is better to cross-link the collagen and incorporate it with bioactive substances for cartilage tissue repair to provide physicochemical and mechanical requirements in the lesion area (Yu et al. 2022).

12.5.1.2 HA

HA is a naturally occurring large molecule that can increase the expression of anti-inflammatory molecules in the chondrocytes. It also stimulates the synoviocytes and also regulates the equilibrium of unbalanced factors. Unmodified HA does not have appropriate mechanical strength. Thus, modification by esterification significantly improves its properties and makes it suitable for cartilage tissue engineering applications. Many studies showed that the injections of HA could reduce knee osteoarthritis symptoms due to its anti-inflammatory and lubricating properties. In the last decade, cell therapy has been introduced as a high potential method for treating cartilage damage. Allogeneic bone marrow mesenchymal stem cells (A BMSCs) promote neocartilage formation, and a matrix containing hyaluronan acts as a carrier for them (Stoop 2008).

12.5.1.3 Fibrin

Fibrin and fibrin glue play important roles as homeostatic agents during the healing of osteochondral defects. Fibrin glue is a fantastic biological vehicle for

chondrocytes due to its excellent cytocompatibility. Some studies confirmed that autologous fibrin glues presented clinically gave satisfactory results compared to commercial ones. Fibrin glue is extensively used to fix cartilage tissue scaffolds in defect sites or render the implant-cartilage junction watertight. However, there has been no determined explanation for its adhesive behavior in cartilage regeneration. Fibrin hydrogel disadvantages include shrinkage, low mechanical stiffness, and high degradability. Chemical modification of fibrin gel can remarkably improve its mechanical properties and solve problems caused by shrinkage. Cross-linking with genipin and combining fibrin with synthetic biodegradable polymers enhance mechanical properties and promote cell adhesion/proliferation and migration (Li et al. 2015).

12.5.1.4 CS

CS is one of the most frequently used natural polymers for cartilage tissue due to its biocompatibility, biodegradability, antimicrobial and anti-inflammatory activities, hemostatic properties, and mucoadhesive nature. Poor mechanical properties of CS can be improved via cross-linking with glutaraldehyde, formaldehyde, and epoxy compounds. These chemical agents have high toxic effects on cells during their release from the structures. Therefore, much attention is focused on using naturally derived cross-linking agents with high cytocompatibility, such as genipin. Genipin is antidiabetic, antibacterial, anti-inflammatory, anticancer, biocompatible, and stable. A genipin cross-linked structure maintains its chemical composition while mechanical strength and Young's modulus are significantly improved. Crosslinked CS hydrogels are an amazing tool for encapsulating MSCs and enhancing cell adhesion, proliferation, migration, and differentiation. Lately, glutamic acid and 1,5-pentanediol mixture have been introduced as the CS cross-linking agents to produce hydrogels. Modifying the hydrogels with *Tilia platyphyllos* extract improves their antioxidant properties in cartilage regeneration and drug delivery systems (Piątkowski et al. 2018). The biodegradation rate of CS depends on the degree of deacetylation (DD) and molecular weight; with the increase of DD, the degradation rate decreases accordingly. Freeze-drying, freeze-gelation, electrospinning, 3D printing, porogen leaching, and gas-forming are the common and frequently used techniques for fabricating cartilage tissue scaffolds. Scaffolds' pore size, porosity, and homogeneity significantly affect tissue healing. Many studies showed that pores smaller than 50 μm make mechanical strength for cartilage tissue scaffolds but increasing the pore size provides more space for chondrocytes. For chondrocyte ingrowth, optimum pore size is in the range of 70–120 μm . Dynamic culture systems can enhance the circulation of a medium within scaffolds cultured for in vitro new cartilage tissue formation. Porous sponges and nonwoven fiber mesh are the most common scaffold architectures appropriate for cartilage tissue engineering. It has been confirmed that TGF- β_3 is more potent than TGF- β_1 in stem cells differentiation into chondrocytes due to its ability to produce collagen type II. Type I and type II BMP-receptors seriously affect cell chondrogenic differentiation pathways (Huang et al. 2020).

12.5.1.5 Agarose

Due to its controlled self-gelling features, solubility in water, electrical neutrality, and controllable mechanical properties, agarose has been extensively used in tissue engineering. It is insoluble in cold water but dissolves readily in hot water. Changing its concentration is the best method for controlling the agarose gel pore size. Agarose can be considered an attractive tool for cartilage regeneration since its stiffness, functional groups, and adjustable water absorption ability provide an appropriate microenvironment for chondrocytes and MSCs. Using agarose-based scaffolds for cartilage repair does not change chondrocyte phenotype and accelerates the precipitation of proteoglycan and glycosaminoglycans. In recent years, agarose has been used for human chondrocyte encapsulation; the results showed that the engineered structures were significantly similar to native tissue. Their compressive Young's and dynamic moduli were 250 and 950 kPa (Cigan et al. 2016). In a study, the effect of agarose concentration on chondrocyte behavior was investigated. The results demonstrated that the matrix formation around the chondrocytes strongly depends on the agarose concentration. Proteoglycans diffusion was improved at low agarose concentration. The following results were observed respectively at 1%, 2%, and 3% agarose concentrations: collagen II deposition and formation of a dense layer of collagen near the cells. It can be concluded that, at a low concentration of agarose, extracellular matrix deposition occurs easily and rapidly, which enhances mechanical properties. The combination of agarose with other biomaterials remarkably improves cellular behavior; for example, the agarose-fibroin composite scaffold can increase the deposition of collagen and sulfated glycosaminoglycans, which shows no changes in chondrocytes phenotype (Singh et al. 2016). Platelet-rich plasma (PRP)-agarose composite scaffold (Yin et al. 2014), poly (ethylene Glycol)-agarose scaffold (Rennerfeldt et al. 2013), and magnetic fibrin-agarose hydrogel (Bonhome-Espinosa et al. 2020) are some of the most frequently used structures in cartilage tissue engineering. It should be noted that many clinical studies must be done to produce the optimum agarose-based composites for cartilage repair (Salati et al. 2020).

12.5.1.6 Alginate

This natural polymer is considered a fantastic tool for cartilage regeneration due to its biocompatibility, biodegradability, and low cost. It can form the gels, and many factors such as molecular weight, gel-forming kinetics, and composition significantly affect alginate porosity, mechanical strength, swelling and degradation rate, and biocompatibility of gel. There are various methods to produce a strong structure with an adjustable degradation rate that chemical cross-linking techniques such as photo-cross-linking, shape memory, click reaction, and enzymatic cross-linking show satisfactory results. Alginate-based porous scaffolds with suitable pore sizes improve cell adhesion and migration. Hydroxyapatite and gelatin microspheres enhance alginate gel's mechanical strength, swelling rate, and bioactivity (Yan et al. 2016). Cartilage tissue cannot repair itself quickly due to insufficient blood vessels, nerves, and lymph. Therefore, cell delivery to the lesion area can accelerate new tissue formation. Direct injection of cells into the target area is unsuitable due to

Table 12.1 The main differences between 3D bioprinting methods (Huang et al. 2021)

	Inject	Extrusion	Laser-based	Stereolithography
Printer cost	Low	Medium	High	Low
Print speed	Quick	Slow	Medium	Quick
Ink viscosity	3.5–12 mPa/s	6×10^7 mPa/s	1–300 mPa/s	Unlimited
Most used	Collagen	Alginate	Collagen	Hyaluronic acid
Materials for	Alginate	GelMA	Matrigel	Alginate
Bioprinting	Poly(caprolactone) (PCL)	PEGDA	ECMs	Poly(ethene glycol) (PEG)
Formation time	Medium	Low	Long	Medium
Cell density	Low	Unlimited	Medium	Medium
Cell viability	85%	40–95%	85%	>85%
Resolution	10–50 μ m	100 μ m	10–100 μ m	200 nm–6 μ m

unwanted cell migration from the defect site, extensive cell death during injection, and lack of support of cell behavior by the tissue around the lesion area. Using hydrogels as a cellular carrier has attracted lots of attention to solve these problems. Alginate-based hydrogels are appropriate carriers for cell delivery due to their excellent biological and physical properties, but cell adhesion properties are so poor; therefore, chemical and physical modifications of alginate have been done. Many studies showed two kinds of frequently used stem cells for cartilage regeneration, MSC and pluripotent such as ESC and iPSCs. In recent years, 3D bioprinting using cells and biomaterials has allowed the fabrication of smart and complex structures that mimic real organs' functions. This technique can overcome the limitations of scaffold-based cartilage tissue regeneration methods. Various 3D printing strategies have been used for cartilage tissue, such as inkjet bioprinting, extrusion-based bioprinting, laser-assisted bioprinting, and stereolithography-based 3D bioprinting (Farokhi et al. 2020). In Table 12.1, the differences between each of these methods are presented.

12.5.1.7 Bacterial Cellulose (BC)

Many nonpathogenic bacteria can produce BC as an exopolysaccharide with unique physical, mechanical, and biological properties such as nontoxicity, high water holding capacity, high porosity, flexibility and molding, exceptional mechanical strength, and suitable interaction with cells. These properties allow BC to be used not only as a scaffold for cells but also to induce and accelerate new tissue formation and improve cell adhesion, migration, and proliferation. It has been mentioned that chondrocytes do not divide, and the cartilage healing process is very long; BC as a scaffold can improve chondrocyte growth and proliferation. The porous structure of BC allows cells to be placed on the surface and inside the scaffold. It cannot be biodegraded in vivo due to the lack of cellulase, which provides enough time for

cartilage repair (Nemati and Gholami 2021). BC has severe disadvantages for tissue engineering applications, such as poor cell interaction, low degradation rate, and lack of antimicrobial properties. To overcome these limitations, *in situ* modifications (adding CS, gelatin, hydroxyapatite, and other bioactive materials to BC) and *ex situ* modifications (cross-linking reactions and homogenization of BC with additive material) are so helpful. Despite much research about using BC for cartilage tissue engineering, cell–BC interactions are complex and need further clinical studies (Gorgieva and Trček 2019).

12.5.1.8 Chondroitin Sulfate (ChS)

ChS is a naturally occurring substance in the body's connective tissues, such as cartilage. It can significantly change the metabolism of chondrocytes, synoviocytes, and other cells involved in cartilage diseases. ChS reduces the NF-KB nuclear translocation, $\text{IL-1}\beta$, interferon (IFN)- γ , TNF- α , the expression of nitric oxide synthase (NOS)-2, and cyclooxygenase (COX)-2. Many clinical studies confirm that ChS has pro-anabolic and anticatabolic effects on chondrocytes; this means that it increases proteoglycan and type II collagen synthesis and inhibits the degradation of the HA. The combination of ChS with supporting materials such as carbon nanotube, graphene oxide, and synthetic polymers induces cartilage regeneration. ChS-based cross-linked hydrogels are extensively used for cartilage repair; for example, methacrylate ChS produces a robust hydrogel by using ultraviolet irradiation, which accelerates new tissue formation (Wang et al. 2007). In another study, tyramine-modified ChS hydrogel was cross-linked with horseradish peroxidase and hydrogen peroxide, then applied for cartilage regeneration. The *in vitro* results showed that the elastic modulus of the hydrogel was insufficient for cartilage. Therefore, further cross-linking must be done to achieve suitable mechanical properties matched with the native tissues (Chen et al. 2016). Based on the *in vitro* studies, combining the ChS and hyper-branched multifunctional PEG copolymer produced an injectable hydrogel for cartilage tissue repair. Its mechanical properties and degradability were improved by cross-linking with thiol-ene reaction. Rat adipose-derived stem cells were loaded onto hydrogel and accelerated chondrogenesis remarkably. This hydrogel with anti-inflammatory ability enhanced endogenous cells migration to the lesion area to active healing process (Li et al. 2021). ChS interacts with its receptors such as CD44, ICAM1, RHAMM, and TLR-4 and activates them, then stimulates the production of the IL-1 receptor, which stimulates MMP-3, MMP-9, MMP-13, and COX-2. In addition, the interaction between ChS and integrins increases the expression of TGF- β 1 and the production of hyaluronic acid. ChS, due to its binding with receptors, can be used as an effective drug delivery tool for chondrogenesis (Gul et al. 2021).

12.5.2 Synthetic Materials

Poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymer, poly(lactic-co-glycolic acid) (PLGA), are synthetic polymers with suitable physical, mechanical,

and biological properties for the regeneration of cartilage tissue. Currently, PLA and PGA have been successfully used as sutures, screws, pins, and treating cartilage defects in rabbits (Duan et al. 2021). PEG, a biocompatible synthetic polymer, is extensively used with other intelligent and bioactive materials to improve their mechanical features for cartilage repair. Many *in vitro* and *in vivo* studies confirmed that PEG-based hydrogels promoted MSCs differentiation to chondrocytes. PCL, due to its nontoxic characteristics and appropriate mechanical strength, is frequently used as a scaffold and as a coating on the scaffold for cartilage regeneration and promotes chondrogenic differentiation of MSCs. Polyvinyl alcohol (PVA), a biodegradable and biocompatible polymer, is used for cartilage regeneration due to its hydrophilic nature, high water absorption ability, and suitable elastic and compressive properties and osteochondral defects. Cross-linking of PVA-based structures is necessary to achieve an adjustable biodegradation rate (Huang et al. 2019).

12.5.3 Bioactive Molecules Used for Cartilage Tissue Engineering

There are two kinds of materials that are used as bioactive molecules for cartilage tissue engineering (a) small molecular weight bioactive compound, and (b) high molecular weight materials (the combination of natural and synthetic materials). These molecules participate in tissue regeneration and play an essential role in interacting with cells (Huang et al. 2019).

12.5.3.1 Kartogenin (KGN)

KGN, a small bioactive molecule, promotes MSCs differentiation to chondrocytes. It breaks the core-binding factor β (CBF β) and filamin A to improve the formation of the CBF β -RUNX1 complex and increase the aggrecan and collagen II expression. KGN transforms TGF- β signaling pathway to the lesion area and accelerates new tissue formation. Many clinical studies showed that KGN remarkably decreased nitric oxide production, glycosaminoglycans (GAGs) secretion, and inhibited ECM collapse. It can also maintain the chondrocyte phenotype.

It should be noted that KGN can easily be cleaned in the body, so the direct injection of it has no effects on the healing process. KGN in the unwanted area can cause an overgrowth of normal cells and tissues. Therefore, it must be loaded in drug carriers. The Carboxyl group in KGN can strongly bind to the amino group in CS to enhance KGN pharmacokinetics. PEG-modified poly(amidoamine) dendrimer is another amazing carrier for KGN, significantly increasing the expression of SOX-9, aggrecan, and collagen II (Hu et al. 2017). In a study, thermoresponsive nanospheres were used as carriers of KGN for new cartilage formation and chondroprotection and diclofenac for preventing inflammation. The *in vitro* results showed the pattern of drug release depended on temperature, diclofenac released so quickly, and KGN so slow (Kang et al. 2016).

12.5.3.2 Simvastatin

Simvastatin belongs to a group of drugs called HMG CoA reductase inhibitors, or “statins,” it is used together with a proper diet to treat high cholesterol and triglyceride levels in the blood. It can prevent many diseases caused by clogged arteries and reduces mortality in heart diseases. In addition, simvastatin inhibits matrix metalloproteases (MMPs) expression, interleukin-1 β expression, and reactive oxygen species production. Statins also prevent MSCs differentiation into chondrocytes and osteoblasts. They significantly increase the mortality rates of MSCs and reduce their ability to repair DNA. Many studies show that statins are anti-inflammatory and modulate an immune response. Statins repress the Class II Major Histocompatibility Complex (MHC-II) expression induced by IFN-gamma, and the decrease in MHC-II expression affects the activation of CD4⁺ T lymphocytes. Statins suppress microRNA and inhibit tumor growth by stopping blood supply (Huang et al. 2019).

12.5.4 Smart Biomaterials for Cartilage Tissue Regeneration

In recent years, piezoelectricity has gained its application in tissue regeneration, especially at the site where the collagen polymer is used. Piezoelectricity is the ability of materials to develop an electric charge in response to mechanical stress. Many studies revealed that electrical stimulation produced TGF- β , which can accelerate cell proliferation and differentiation. A piezoelectric polymer such as poly (vinylidene fluoride), a copolymer of vinylidene fluoride (VDF) and trifluoroethylene (TrFE), poly-3-hydroxybutyrate-3-hydroxy valerate (PHBV), polyamides, poly-L-lactic acid (PLLA), cellulose, collagen and chitin, and piezoelectric ceramic such as barium titanate (BT), zinc oxide (ZnO), potassium sodium niobate (KNN), lithium sodium potassium niobate (LNPN), and boron nitride nanotubes (BNNT) and their composites are frequently used materials for manufacturing smart cartilage scaffolds. These scaffolds have strong positive effects on the adhesion and proliferation of chondroblast cells and improve MSCs differentiation into chondrocytes. Additionally, piezoelectric scaffolds demonstrate a functional improvement of cells without needing bioactive molecules and drugs (Jacob et al. 2018).

Magnetic response hydrogels are advanced nanocomposite hydrogels used widely in tissue engineering to enhance cell interaction and behavior. Ferric oxide-containing magnetic hydrogel can prevent the progression of breast tumors (Gao et al. 2019). The combination of iron-oxide nanoparticles and polymers produces advanced magnetic composites; for example, γ -Fe₂O₃, Fe₃O₄, and CoFe₂O₄ are the most commonly used nanoparticles for fabricating magnetic hydrogels for cartilage repair. Conductive gold nanoparticles, silver antibacterial nanoparticles, and TiO₂ nanoparticles are doped with polymers and improve mechanical strength, degradation rate, and loading capacity, and accelerate cell growth during the cartilage healing process (Kumar 2018; Barrow et al. 2018). Nonmetallic nanoparticles such as hydroxyapatite, silica, calcium phosphate, and silicate β -Wollastonite nanoparticles are attractive for cartilage tissue engineering due to their large surface

area, suitable mechanical properties, and good biocompatibility. Some studies showed that the mechanical strength of hydrogels could be improved by adding mesoporous silica and sodium-calcium bentonite.

Additionally, these compounds presented excellent interaction with cells and antibacterial properties (Bonifacio et al. 2020). Lately, exosomes, the nano-sized vesicles with a diameter of 50–130 nm, have several roles in various biological processes. They can be modified for drug delivery. Exosomes from stem cells have strong therapeutic effects on cartilage diseases due to their stability, available resources, and easy processability. Despite the many advantages of these structures, there are still serious challenges in their clinical application, such as how to use exosomes to accelerate cartilage repairing, enhance their antimicrobial properties and increase their therapeutic effects, and load drugs in exosomes' hollow structure. It can be concluded that more *in vivo* studies must be done to understand the answers to all these questions (Liu et al. 2019).

12.6 Conclusion and Perspective

Using bioactive molecules, cells and scaffolds is the main strategy to produce new cartilage tissue. Significant advances in material science and manufacturing methods have made it possible to achieve more effective compounds for the treatment of cartilage diseases, which can overcome the limitations of conventional materials. Cartilage repairing techniques are still developing, and the number of debatable parameters in cartilage tissue engineering are very large. Each of them needs to be examined carefully and separately. Incorporating various sciences such as material science, microbiology, molecular and cellular biology, and polymer engineering can significantly increase the chances of success. The demand for more advanced structures with excellent mechanical, physical, and biological properties is increased with the in-depth study of cartilage tissue components and the surrounding environment. Due to their unique features, nanomaterials and hydrogels can be introduced as amazing tools for cartilage regeneration. Many studies confirmed that growth factors (GFs) supported new tissue formation, but several challenges must be solved before using them. For example, GFs concentration in the target tissue must be known since it severely affects cell migration and attachment. It should be noted that cartilage is a complex tissue and is affected by several GFs. At the same time, in many studies, one or two GFs have been used, and it is almost impossible to study the simultaneous effect of all GFs, so the results of *in vitro* studies will not be completely matched with the function of the natural tissue. All treatments for cartilage diseases have certain limitations. The treatment result was more successful for large lesions ($>4.5\text{ cm}^2$) than for small damages. To date, none of the treatments have been able to recreate native hyaline cartilage due to poor matrix properties. Combining several therapeutic strategies can significantly accelerate damaged tissue healing. Gene delivery is one of the most important treatment methods that is hoped to be used for clinical applications in the future. Among the wide range of materials for gene transferring, polymeric biomaterials are highly regarded and show satisfactory

results, but their efficiencies are still very low compared to viral transmitters. One of the important challenges in gene delivery with polymeric materials is the effective dose for the healing process and how to release it in the desired area, the answer to which will require more detailed studies. Lately, piezoelectric electrospun scaffolds have been used for cartilage tissue engineering. These complex structures with excellent spatial resolution and inimitable properties can mimic the mechanical and biophysical properties of the ECM. Many studies revealed that piezoelectric fibrous scaffolds remarkably improved MSCs differentiation to chondrocytes and increased chondrogenic gene expression. The main limitation of piezoelectric structures is the nonbiodegradability of piezoceramics, while biodegradable piezoelectric biomaterials such as PLLA have poor mechanical strength. Nonbiodegradable structures support new tissue formation for long periods and remain intact. Therefore, additional surgery is needed to remove them after tissue regeneration, but this surgery will damage the new tissue. Nonbiodegradable piezoelectric scaffolds can be used for disease modeling and as coating structures to enhance their interaction with cells. Smart or sensitive materials that respond to external stimuli are extensively used as cartilage scaffolds. Photoresponsive and temperature-sensitive materials can easily change their solubility, degradability, and biological properties by applying light and temperature stimuli. These stimuli can be used in a special region without contact; therefore, their doses will be adjusted to control the responses. Visible, ultraviolet (UV), and near-infrared (NIR) lights are frequently used in tissue engineering. While UV light is strong and highly toxic, visible light shows high tissue penetrability, and NIR light is not toxic and presents high tissue penetration. Due to their poor mechanical properties, poor cell interaction, and toxicity, electroresponsive materials are combined with natural and synthetic polymers for cartilage regeneration. Humidity-responsive materials can show some unique properties by absorption or desorption of moisture and return to their original form after removing the stimulant. These smart materials have wide advantages and drawbacks, but new materials and new manufacturing methods can remove these limitations.

Acknowledgments Part of this research was supported by National Institute of Dental & Craniofacial Research of the National Institutes of Health under award number R15DE027533, R56DE029191, and 3R15DE027533-01A. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Importance of 3D Printing Techniques in Cartilage Tissue Engineering

13

Sharareh Mahdavi and Shohreh Mashayekhan

Abstract

3D bioprinters have revolutionized tissue engineering and regenerative medicine approaches. This chapter addresses various 3D printing techniques including both scaffold-based and scaffold-free approaches implemented in cartilage tissue engineering. Recent studies have been reviewed and scaffold-based 3D printing techniques have been divided into five main categories as extrusion/microextrusion, inkjet, in-situ, laser-based, and stereolithography. The differences, advantages, and disadvantages of each approach have been argued. Furthermore, the effect of different biomaterials and cell types on controlling cellular behavior has been explored. In addition, the impact of using different bioinks on in vitro/in vivo results has been explained.

Keywords

Tissue engineered scaffold · 3D printing techniques · Extrusion-based 3D printers · Inkjet-based 3D printers · Laser-based 3D printers · Stereolithography

13.1 Introduction

Cartilage is an elastic connective tissue that covers the surface of joints and reduces the friction caused by bone and joint movements (Parvizi and Kim 2010). Furthermore, it provides support, helps maintaining organ shape, and absorbs shock (Chiu

S. Mahdavi

Research Operations, The Hospital for Sick Children, Toronto, ON, Canada

S. Mashayekhan (✉)

Department of Chemical and Petroleum Engineering, Sharif University of Technology, Tehran, Iran

e-mail: mashayekhan@sharif.edu

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M. Baghaban Eslaminejad, S. Hosseini (eds.), *Cartilage: From Biology to Biofabrication*, https://doi.org/10.1007/978-981-99-2452-3_13

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and Waldman 2016). The uniformity and dense structure of cartilage tissue is caused by dense matrices of collagen type II fibers (Wang et al. 2022). The extracellular matrix (ECM) of cartilage tissue is mainly secreted by chondrocytes cells. This matrix largely contains proteoglycans and consists of collagen and elastic fibers (Bonyan et al. 2018). Cartilage tissue is mainly divided into three types of hyaline, elastic and fibrocartilage based on their structure and functionality (Parvizi and Kim 2010; Naumann et al. 2002). The first one, hyaline, is the most abundant type in human body and it is the main part of articular cartilage (Francis et al. 2013). About 90–95% of hyaline matrix is composed of collagen type II (Naumann et al. 2002; Roberts et al. 2009; Gartner 2021). The tissue has a bluish color which changes to yellow as it becomes calcified as a part of bone growth, degenerative causes, and aging (Gartner 2021; Chang et al. 2022). Articular cartilage has undeniable role in reducing friction, absorbing shock, and along bones slide over one another smoothly (Gartner 2021; Chang et al. 2022; Azenol and A-zer 2020). Hyaline cartilage presents on the surface of articular joint and in trachea (also known as the windpipe), nasal septum, and different parts of the rib (Chiu and Waldman 2016; Chang et al. 2022). Elastic cartilage is a yellowish tissue that composed of dense network of elastin fibers. This is the main difference between hyaline and elastic cartilage. The protein elastin fibers make it easy for the tissue to return to its original shape after any changes that cause by movement (Parvizi and Kim 2010; Gartner 2021) and it exists in the nonload bearing organs namely nose, external ears, parts of larynx, auditory tubes and epiglottis (Gartner 2021; Chang et al. 2022; Watkins and Mathieson 2009; Standring 2021). Fibrocartilage is a whitish tissue that has a copious amount of collagen type I (Chang et al. 2022). The fibrous texture provides a dense structure and it supports shear, tensile and compressive stress (Chang et al. 2022; Standring 2021). This type of cartilage can be found in tendons, ligaments, menisci, parts of spine and knees (Chang et al. 2022; Standring 2021). Although some mammalian cartilage contains few vessels, it is mostly known as an avascular tissue (Bonyan et al. 2011; Dean 2017). In addition to the lack of blood supply, cartilage tissue does not contain nerves and it is considered an aneural tissue. Since it is also an alymphatic tissue, any pain related to the cartilage body parts is due to the inflammation and sensitivity of the joints and bones associated with that cartilage (Chang et al. 2022; Chung and Burdick 2008; Zhang et al. 2009). All these unique characteristics made it more difficult and time-consuming for this tissue to self-heal and regenerate during any damages and injuries (Chang et al. 2022; Chung and Burdick 2008; Zhang et al. 2009; Niklopoulos et al. 2019). Therefore, due to calcification of this aneural and avascular tissue, cells embedded within the ECM would die eventually (Chang et al. 2022). One of the most conventional techniques for cartilage damage is autograft and allograft implant and in acute cases, it will lead to joint replacement (Zhang et al. 2009; Fuggle et al. 2020). However, all these traditional treatments have the disadvantage of limited donor tissue availability, inflammatory response of the host tissue, infection, and implant failure (Zhang et al. 2009). Hence, tissue engineering and regenerative medicine approaches have attracted a lot of attention in cartilage regeneration (Chiu and Waldman 2016; Francis et al. 2013; Chung and Burdick 2008; Zhang et al. 2009; Niklopoulos

et al. 2019). Various techniques and methods have been developed using the fundamental knowledge of material science and engineering, biology, and chemistry in this emerging multidisciplinary research field (Zhang et al. 2009; Mahdavi and Abdekhodaie 2022; Mahdavi and Mashayekhan 2022; Mahdavi et al. 2020a). The main goal of tissue engineering and regenerative medicine techniques is to resemble the microenvironment of the native tissue for the cells to stimulate the proper signaling pathways (Chung and Burdick 2008; Zhang et al. 2009; Mahdavi and Abdekhodaie 2022; Mahdavi et al. 2020a; Jelodari et al. 2022). This will lead into the secretion of ECM constitutes either by the healthy cells remained in the host tissue or by the delivered cells using bioengineered methods (Chung and Burdick 2008; Zhang et al. 2009; Mahdavi and Mashayekhan 2022; Mahdavi et al. 2020a). In general, tissue engineering and regenerative medicine approaches can be divided into two main categories of scaffold-based and scaffold-free techniques (Mahdavi et al. 2020a). In the case of scaffold-based methods, scaffold has an important role in regulating the cellular behavior. Biocompatibility is one of the critical features of a bioengineered scaffold, which provides proper cell support for cell attachment and growth in *in vitro*, *ex vivo*, *in vivo*, and clinical studies. In *ex vivo*, *in vivo*, and clinical studies, tissue integration alongside with biocompatibility have significant effect on cellular support (Chiu and Waldman 2016; Francis et al. 2013; Chung and Burdick 2008; Zhang et al. 2009; Niklopoulos et al. 2019). Biodegradability is the other vital characteristics when it comes to designing an appropriate scaffold. An ideal scaffold would support cells during cellular growth and differentiation and degrade over time while the new tissue is forming. Mechanical strength of the scaffold should be elaborated precisely to strike a balance between supporting cellular growth and attachment under native mechanical loads and also replacing the engineered scaffold with a newly formed tissue naturally over time. Physical properties of the scaffold such as structure (hydrogel, fibrous, porous) (Chung and Burdick 2008; Niklopoulos et al. 2019; Jelodari et al. 2022; Del Bakhshayesh et al. 2019; Cheng et al. 2019), surface topography (nano/micropatterns) (Griffin et al. 2015; Wu et al. 2017; Janvikul et al. 2013), and two/three dimensional (2D/3D) microenvironment can mimic native tissue ECM for regulating the cells fate (Francis et al. 2013; Chung and Burdick 2008; Niklopoulos et al. 2019; Mahdavi et al. 2020a; Xing et al. 2019). Porosity and pore size are also adjustable to allow nutrients in and wastes out of the microenvironment and efficient cell migration (Zhang et al. 2009; Mahdavi and Abdekhodaie 2022; Mahdavi and Mashayekhan 2022; Mahdavi et al. 2020a, b, 2021; Froushani et al. 2021; Ahmed 2015). Material science and engineering approaches would be applied to adjust scaffold properties for cellular support and attachment. In addition, bioactive molecules can be integrated into the scaffold structure to enhance its characteristics which might compromise the mechanical and chemical properties of the biomaterial (Patel et al. 2019; Kwon et al. 2016; Birru et al. 2020; Lee and Shin 2007; Jooybar et al. 2019). Therefore, the impact of tissue engineering technique which can be applied to fabricate a bioengineered scaffold on tissue regeneration is significant. There are diverse advancing tissue engineering and regenerative medicine strategies for cartilage regeneration such as electrospinning (Yilmaz and Zeugolis 2020; Ding et al. 2021;

Li et al. 2018; Horner et al. 2016; Hernandez-Rangel et al. 2020; Semitela et al. 2020; Chen et al. 2020), nanotechnology-based techniques (Zhang et al. 2021; Ozkan and Yanmis 2019; Eftekhari et al. 2020), microfluidic approaches (Li et al. 2017a; Zhuge et al. 2022; Zou et al. 2022; Goldman and Barabino 2014; Wang et al. 2011), 3D printing and 3D bioprinting (Yang et al. 2022a; Li et al. 2017b, 2021; Cheng et al. 2020, 2021; Ding et al. 2022; Guo et al. 2018; Xu et al. 2022; Huang et al. 2021a, b; Rosenzweig et al. 2015; Hampton 2015; Sun et al. 2020; Tosoratti et al. 2021; Liang et al. 2022; Helgeland et al. 2021; Nakamura et al. 2021; Al Kishtaini 2019), and the combination of these methods (Lopa et al. 2018; De Mori et al. 2018; Nabizadeh et al. 2022). Over the past decades, 3D printing has a significant development in tissue engineering and regenerative medicine. One of the main reasons that this technique has attracted a lot of attentions is that this technology can be used to fabricate a 3D implant or design complex tissue structures using biomaterials combined with cells, growth factors, etc. which overcomes the limitations and disadvantages of other cartilage tissue engineering approaches (Wang et al. 2022; Huang et al. 2021b; Liang et al. 2022). The present book chapter aims to overview the importance and various techniques of 3D printing to treat defected cartilage tissue.

13.2 3D Printing Essentials in Cartilage Tissue Regeneration

The ultimate goal of tissue engineering and regenerative medicine approaches is to regenerate the defected tissue and organ using engineering techniques without the donor tissue requirement (Mahdavi and Mashayekhan 2022; Mahdavi et al. 2020a). In overall, designed scaffold will be investigated in the laboratory-scale after various in vitro studies and an optimized design will be selected for further animal studies. If the fabricated scaffold has successful in vivo regeneration results, it will be transferred to the clinical trials before the large-scale production (Chung and Burdick 2008; Mahdavi and Mashayekhan 2022; Mahdavi et al. 2020a; Huang et al. 2021b). Therefore, a high resolution and repetitive technique should be used for scaffold fabrication. In all 3D printing approaches, computer modeling will be used to design the scaffold structure based on the geometry of the defected tissue. This additive manufacturing or rapid prototyping technique fabricates a computer-designed geometry layer-by-layer and it has progressed with technology advancement (Mahdavi and Mashayekhan 2022; Mahdavi et al. 2020a, b, 2021; Yang et al. 2022a). Scaffold-based 3D printing techniques in cartilage tissue regeneration can be divided into various categories depending on the type of the 3D printer (Yang et al. 2022a; Xu et al. 2022; Huang et al. 2021b; Tosoratti et al. 2021; Liang et al. 2022). Scaffold-free 3D printing have also been applied to regenerate cartilage tissue (Taniguchi et al. 2018; Alblawi et al. 2020; Gopinathan and Noh 2018). In scaffold-based approaches, in addition to the solution viscosity, the surface tension, cross-linking process, and the properties of the solution deposition method impact the printability of the bioink (Gopinathan and Noh 2018). In all of the scaffold-based 3D printing approaches, bioink is an important factor that affects physical, mechanical, and

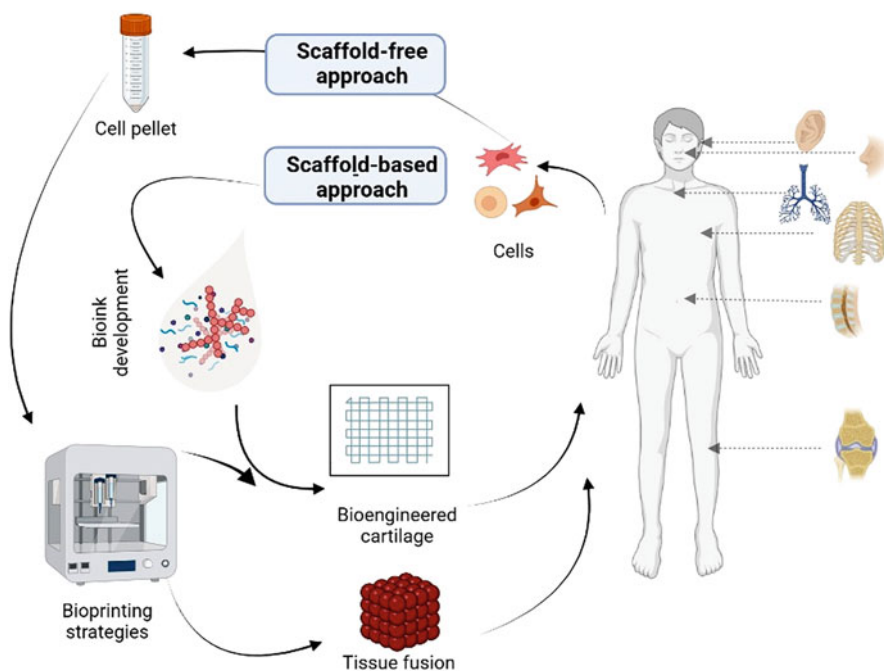


Fig. 13.1 Schematic of cartilage tissue engineering using 3D bioprinting technique. Recreated from (Agarwal et al. 2021) with permission © 2021 Elsevier. All rights reserved

biological properties of the scaffold. Bioinks can be classified into two main categories: scaffold-based and scaffold-free. In scaffold-based methods, the bioink is the blend of the biomaterials that can be combined with the components of the extracellular matrix, growth factors, and living cells. The only difference between 3D printing and 3D bioprinting is that in the latter, bioink also contains living cells (Gopinathan and Noh 2018). In the scaffold-free approaches, living cells are directly deposited in a specific structure into the defected area to form the functional tissue over time (Alblawi et al. 2020; Gopinathan and Noh 2018) (Fig. 13.1). Viscosity of the bioink is one of the important factors which have a significant impact on the printability, biodegradation, and mechanical strength of the 3D-printed sample. Moreover, this property should be tunable so that the proposed bioink solution can be produced in a large scale using various 3D printing approaches (Liang et al. 2022; Gopinathan and Noh 2018; Bishop et al. 2017; Potyondy et al. 2020). While the inkjet-based bioprinters require low-viscosity bioink, high-viscosity prepolymer solution can be 3D printed using the extrusion-based and laser-based 3D printers (Mahdavi and Mashayekhan 2022; Gopinathan and Noh 2018). 3D printing high-viscous bioinks using extrusion or droplet-based 3D printers generate high shear stress during the printing process. This might lead to the biomaterial degradation or cell damage. Therefore, using bioink solutions that have shear thinning properties counterbalances the shear strain (Gopinathan and Noh 2018). Other properties of the

ideal bioinks in 3D bioprinting are as follows (Mahdavi and Mashayekhan 2022; Mahdavi et al. 2020a; Gopinathan and Noh 2018):

- Adequate stiffness should be optimized in such a way that the printed structure remains intact after 3D bioprinting without negatively affecting the cell viability.
- Biodegradability should be tuned with tissue regeneration process so that the 3D bioprinted scaffold is replaced by the secreted ECM over time. Moreover, scaffold degradation should not induce any inflammatory responses as well.
- Biological properties should be adjusted to maximize cell-cell, cell-scaffold, and cell-tissue integrations. This also includes permeation of oxygen and nutrients and excretion of metabolic wastes.

Bioink properties should be tuned in a manner that high-resolution end product can be achieved without having any adverse impact on mentioned requirements. Furthermore, bioink compartments should be economically justified especially for large-scale production. Various biomaterials were used as a bioink in 3D printing approaches. Synthetic biomaterials (poly (ethylene glycol) (PEG), poly lactic acid (PLA), poly caprolactone (PCL), poly-glycolic acid (PGA), etc.) can be polymerized quickly and have high mechanical strength. In addition, their morphological and degradation properties can be easily tuned. However, synthetic materials have lower biocompatibility and biodegradability comparing to natural biomaterials (collagen, agarose, fibrin, gelatin, silk fibroin, etc.). Besides proper cytocompatible properties of natural biomaterials, they can be derived from ECM components and have similar properties to the native tissue. Nevertheless, low mechanical strength and temperature sensitivity of the biomaterials derived from natural resources need to be addressed. Using the combination of both synthetic and natural biomaterials helps merge the advantages of both biomaterials and overcome their limitations (Zhang et al. 2009; Mahdavi and Mashayekhan 2022; Mahdavi et al. 2020a, b, 2021; Jelodari et al. 2022; Huang et al. 2021b; Gopinathan and Noh 2018). Therefore, biomaterials have key role in bioink properties.

In this chapter, the recent 3D printing approaches applied to regenerate cartilage tissue were categorized into two main groups of scaffold-based and scaffold-free 3D printing. Scaffold-based 3D printing has been divided into five subcategories as extrusion/microextrusion, inkjet, in situ, laser-based, and stereolithography. Furthermore, the impacts of 3D printer type and bioink components were also discussed in detail.

13.3 Overview of 3D Printing Approaches in Cartilage Tissue Engineering

Using 3D printing/bioprinting techniques in tissue engineering and regenerative medicine has revolutionized both scaffold-free and scaffold-based approaches for restoring complex tissue structure (Tasnim et al. 2018; Zhang et al. 2017; Dey and Ozbolat 2020; Vermeulen et al. 2017). In the latter, 3D bioprinting operating

procedure involves three important steps: preprocessing, processing, and postprocessing phases (Fig. 13.2a) (Mahdavi and Mashayekhan 2022). In the first step, the geometry of the defected tissue will be recreated using a computer-aided design (CAD) and the format of the file should be identifiable by the 3D printer. In this phase, the fidelity of the created 3D structure and the type of 3D printer have an undeniable impact on the results. In the second step, processing, bioink will be prepared based on the required physical, chemical, mechanical, and biological properties of the host tissue. The selections of biomaterial, cell source, and cross-linking method are the major parts of this phase. The last but not least step is the processing stage which contains in vitro examinations before moving to the next level, i.e., in vivo investigation. Choosing suitable and adequate tests and analyses is the vital part of this phase (Bishop et al. 2017; Potyondy et al. 2020). Some of the fascinating recent studies that used 3D printing approaches for cartilage tissue engineering have been reviewed in the following section.

13.3.1 Scaffold-Based 3D Printing Approaches

3D printing has made it possible to fabricate novel cartilage tissue engineering scaffolds with tailored geometry and shape, macro/microstructure, mechanical strength, cellular response (Wang et al. 2020). In the inkjet-based bioprinter, 3D-designed geometry forms with multiple layers of deposited droplets. Thermal, electric or electromagnetic forces will be applied to the bioink solution to form droplets in the form of liquid. Inkjet-based 3D printers are easy to use, economic, and allow the delivery of small volume of the bioink to the nozzle. Nevertheless, it requires low-viscosity prepolymer solutions which results in low 3D printing resolution in many cases and limits its application. Furthermore, this limits biomaterial selection to the solutions that only generate droplets in the form of liquid. In thermal-induction inkjet-based 3D printers, the liquid droplets will be heated which requires biomaterials that do not degrade with heating to the specific temperature. This also affects the cytocompatibility in 3D bioprinting approaches (Huang et al. 2021b; Bishop et al. 2017; Zhang et al. 2019). To address some of the disadvantages of inkjet-based 3D bioprinters, extrusion/microextrusion-based 3D bioprinters are introduced to fabricate 3D scaffolds (Bishop et al. 2017; Zhang et al. 2019). In extrusion/microextrusion-based 3D bioprinters, high-viscosity bioink containing high cell density will be used to form a 3D hydrogel with the designed structure. In this 3D printers, a piston, pneumatic pump or screw distribute hydrogel filaments through the nozzle onto the substrate. Hence, in extrusion-based 3D printers, wider variety of biomaterials can be used with higher printing speed. The ability of using high-viscous bioinks allows the users to have higher printing resolution and more precise-shaped scaffolds. However, the shearing force applied to the bioink on the nozzle wall reduces cell viability and causes cell dehydration (Huang et al. 2021b; Bishop et al. 2017; Tarassoli et al. 2018). To eliminate nozzle-related difficulties, laser-based 3D printers are developed which use laser beam to transfer the configured geometry to the bioink. In these 3D printers, a gold or titanium layer

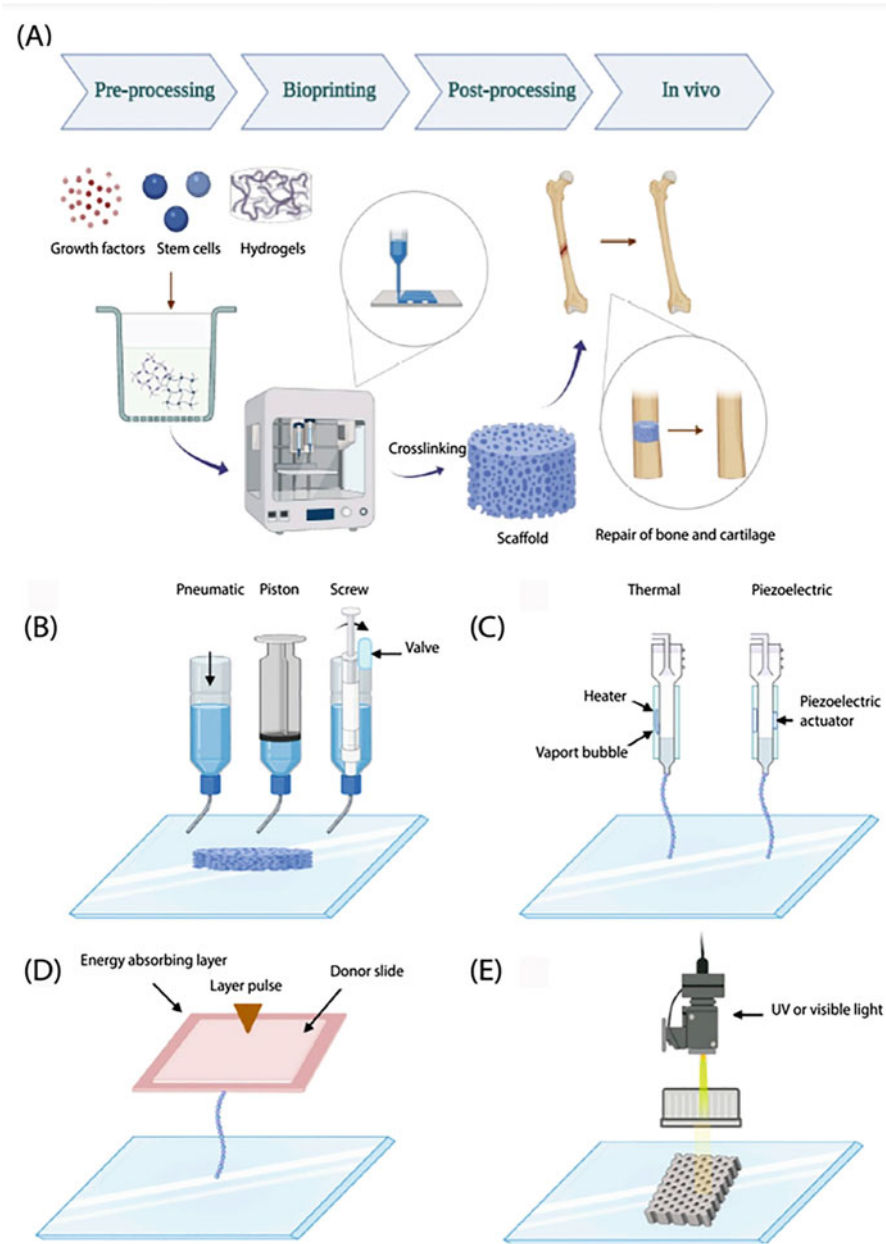


Fig. 13.2 3D printing systems and bioink characterizations. (a) Schematic of various steps of 3D printing from preprocessing to postprocessing. Schematic illustrations of 3D printing systems. (b) Extrusion-based. (c) Inkjet-based. (d) Laser-assisted. (e) Stereolithography. Adapted from (Yang et al. 2022b) licensed under creative commons license

absorbs the laser energy and transfers it into the bioink solution to fabricate a bioengineered scaffold. Since the disadvantages of nozzle-based printers such as nozzle clogging and cell damaging caused by the shear stress-induced forces are not applied in this method, bioink solutions with a wide variety of viscosity range can be used. Using laser beam to transfer the designed geometry to the prepolymer solution provides high special resolution and makes it possible to go up to sub-micron resolution which is a great advantage in fabricating scaffolds with complex structures. Despite of all these positive characteristics, these 3D printers require expensive hardware and software which reduces their application in cartilage tissue engineering (Huang et al. 2021b; Bishop et al. 2017). Another 3D bioprinting method to produce high-resolution hydrogels is stereolithography-based 3D bioprinters. In this technique, either ultraviolet (UV) light or visible light is used to cross-link a photo-cross-linkable-bioink solution in a layer-by-layer process. It can be used to construct scaffolds with a precise geometry; however, the minimum size that can be achieved using this method is limited to the width of a beam of the light. Moreover, it limits the bioink selection to the biomaterials that can be photopolymerized. Usage of UV light can cause cell damage and mutations. Nevertheless, this technique is widely used in tissue engineering because it is highly cytocompatible (Mahdavi et al. 2020b; Huang et al. 2021b; Bajaj et al. 2014). All the techniques mentioned above are applied to generate a prefabricated scaffold for cartilage tissue engineering (Fig. 13.2b–e).

Recently, *in situ* 3D bioprinting technique has attracted scientists' attention in tissue engineering and regenerative medicine approaches. In this 3D bioprinted, postprocessing process is not required and hydrogel filaments will be deposited directly into the defected area while being photo-cross-linked *in situ*. In general, this technology developed with the combination of nozzle-based and stereolithography techniques (Li et al. 2017b; Galarraga et al. 2019a; Di Bella et al. 2018a). The advantages and disadvantages of various 3D printing techniques were listed in Table 13.1 below.

In the following section, various studies using 3D printing approaches for cartilage tissue engineering were reviewed. List of various scaffold-based 3D printing techniques used for treating cartilage defects are summarized in Table 13.2.

13.3.1.1 Inkjet-Based 3D Printers

In the inkjet-based bioprinter, external forces will be used to transform bioink solution into droplets (Huang et al. 2021b). The impact of using an inkjet-based 3D printer to construct a hydrogel comparing to the conventional photo-cross-linking method was studied. PEGDA hydrogel containing human chondrocytes was deposited layer-by-layer using an inkjet 3D printer and each layer got cross-linked with UV light simultaneously during the printing process. The biological properties were compared to the hydrogel constructed using just a UV light and without implementing a 3D printer. It was reported that the ability to deposit the bioink in the layer-by-layer manner helped distributing cells through the final structure whereas cells sank into the bottom of the hydrogel fabricated with a conventionally photo-crosslinking procedure. Implementing a 3D printer was

Table 13.1 Comparison of scaffold-based 3D printing techniques

3D printing technique	Nozzle-based	Advantages	Disadvantages	Ref.
Inkjet	√	– Easy to use – Economic	– Requirement of low-viscosity bioink – Low printing resolution – Nozzle clogging – Limitation in biomaterial selection – Cell damage at the nozzle	Huang et al. (2021b); Bishop et al. (2017); Zhang et al. (2019)
Extrusion/microextrusion	√	- Can be used for high-viscosity bioink - High printing resolution	- Nozzle clogging - Cell damage and dehydration at the nozzle	Huang et al. (2021b); Bishop et al. (2017); Tarassoli et al. (2018)
Laser-based	×	- Elimination of nozzle difficulties - High printing resolution	Expensive hardware and software	Huang et al. (2021b); Bishop et al. (2017)
Stereolithography	×	- Elimination of nozzle difficulties - High printing resolution - High cell viability	- Limitation in biomaterial selection - Cell damage in the case of UV light	Mahdavi et al. (2020b); Huang et al. (2021b); Bajaj et al. (2014)
In situ	√	- Elimination of postprocessing step - High cell viability	- Nozzle clogging - Limitation in biomaterial selection	Li et al. (2017b); Galarraga et al. (2019a); Di Bella et al. (2018a)

observed to increase the crosslinking speed which improved cell viability significantly (Cui et al. 2014). This was also observed in 3D bioprinted PEGDMA samples. The 3D bioprinted hydrogels were photo-polymerized during and after the 3D printing process. It was reported that in the latter, samples had to be exposed to the UV light for at least 10 min to get cross-linked in the postprinting process. This dropped cell viability from around 90% to 65%. The impact of the substrate on which the 3D bioprinted samples were deposited was also investigated on GAG/DNA and collagen type II/DNA contents. Osteochondral (OC) plug harvested from bovine femoral condyles and a plastic mold were used as the biopaper for

Table 13.2 Scaffold-based 3D printed constructs for cartilage regeneration

3D printing method	Biomaterial	Cross-linking method	Cell type	Experimental phase	Cartilage type	Regeneration outcome	Ref
Inkjet-based	PEGDA	UV light photo-cross-linking (I-2959 photoinitiator)	Human chondrocytes	In vitro	Articular cartilage	- Homogenous cell distribution in 3D bioprinted hydrogel - Cell viability of 90% after the printing process - Increase in proteoglycan production over time within 4 weeks	Cui et al. (2014)
Inkjet-based	PEGDMA	UV light photo-cross-linking (I-2959 photoinitiator)	Human chondrocytes	Ex vivo (Osteochondral (OC) plug harvested from bovine femoral condyles)	Articular cartilage	- Cell viability of about 90% in the photo polymerized samples process during the 3D bioprinting - Increase in GAG/DNA and collagen type II/DNA content over time from 2 to 4 weeks	Cui et al. (2012)
Inkjet-based	Alginate	UV light photo-crosslinking (I-2959 photoinitiator) and ionic cross-linking with CaCl ₂	Human adipose-derived mesenchymal stem cells and murine fibroblasts (NIH-3T3-GFP)	In vitro	Articular cartilage	Increase in cell viability by 20% by UV photopolymerization during the 3D bioprinting process instead of cross-linking the hydrogel after 3D bioprinting	Raddatz et al. (2018)

(continued)

Table 13.2 (continued)

3D printing method	Biomaterial	Cross-linking method	Cell type	Experimental phase	Cartilage type	Regeneration outcome	Ref
Inkjet-based	PCL/fibrin/collagen	Thrombin	Rabbit ear cartilage chondrocytes	In vitro and in vivo (mice model)	Articular cartilage	- Cell viability of 82% - Cartilage-like tissue formation in the animal models after 2 months	Xu et al. (2013)
Extrusion-based	GelMA/alginate and β -tricalcium phosphate microparticles	UV light (photoinitiator Igacure 2959) and CaCl_2	Human mesenchymal stem cells	In vitro	Articular cartilage	Induction of ECM protein and alkaline phosphate expression with the presence of ceramic microparticles	Kosik-Kozio et al. (2019)
Microextrusion	Gelatin/hydroxyapatite (HAP)	Physical (thermoreponsive properties of gelatin) and enzymatic cross-linking (trans-glutaminase)	Human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSC)	In vitro and In vivo (pig model)	Articular cartilage	- Cell viability of more than 75% - Generation of cartilage-like after 6 months in pig models having knee cartilage cavities	Huang et al. (2021a)
Extrusion-based	PCL and starch	CaCl_2 solution	Human adipose tissue-derived stem cells (hADMSCs)	In vitro	Articular cartilage	- No difference in cell proliferation between hydrogels containing beta cyclodextrin (β-CD)-modified alginate and samples incorporated with unmodified alginate - Improvement of GAG content in β-CD-modified alginate hydrogels	Mohseni et al. (2022)

Extrusion-based	Hyaluronic acid (HA)/alginate/poly(lactic acid) (PLA)	CaCl ₂ solution	Human chondrocytes	In vitro	Hyaline cartilage	- Cell viability of 85% postprinting. Increase in hyaline cartilage-specific genes with the presence of HA - Augmentation of collagen type II and GAG in the samples containing HA	Antich et al. (2020)
Extrusion-based	Methacrylated poly [N-2-hydroxypropyl) methacrylamide mono/diacrylate] (pHPMA-lac)/poly ethylene glycol (PEG)/methacrylated hyaluronic acid (HAMA)/polycaprolactone (PCL)	UV light (Igacure 2959)	Horse chondrocytes	In vitro	Articular cartilage	- Increase of fibrocartilage tissue-specific gene markers expression with higher HAMA concentration - The expression of hyaline tissue-specific gene markers in the samples containing various cell donors with the presence of HAMA	Mouser et al. (2017)
Microextrusion	Alginate/GelMA/chondroitin sulfate (CS) and graphene oxide (GO) nanofiller	UV light (photoinitiator 2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropionophenone)	hADMSCs	In vitro	Articular cartilage	- Cell aggregation with high concentration of GO - Expressions of collagen type II, aggrecan, and SOX 9 in samples containing GelMA and CS after 28 days	Olate-Moya et al. (2020)

(continued)

Table 13.2 (continued)

3D printing method	Biomaterial	Cross-linking method	Cell type	Experimental phase	Cartilage type	Regeneration outcome	Ref
Microextrusion	Poly(N-2-hydroxypropyl) methacrylamide-mono/dilactate-polyethylene glycol (pHPMAlac-PEG)/methacrylated chondroitin sulfate (CSMA)	UV light (Igacure 2959)	ATDC5 cells	In vitro	Articular cartilage	-Lower cytocompatibility comparing to scaffolds containing pure pHPMAlac-PEG and pure CSMA after 7 days	Abbadessa et al. (2016)
Extrusion-based	Collagen	Physical crosslinking	Bovine primary fibrochondrocytes	In vitro	Fibrocartilage	- Cell viability of more than 90 % after 10 days in all the samples	Rhee et al. (2016)
Extrusion-based	Silk fibroin/gellan gum/fibrinogen (GG/FB)	UV light (Igacure 2959) and thrombin	Porcine meniscus cells (pMCs)	In vivo (mice models)	Fibrocartilage	- Induction of cartilage regeneration in mice models after 10 weeks	Costa et al. (2020)
Laser-assisted	PCL/hydroxyapatite nanoparticles	N/A	Rat mesenchymal stem cells	In vitro and In vivo (rabbit models)	Articular cartilage	- Regeneration of subchondral bone and articular cartilage in animal models treated with the multilayer scaffold after 12 weeks	Du et al. (2017)
Laser-assisted	PCL	N/A	Porcine chondrocyte cells	In vitro and in vivo (pig models)	Articular cartilage	- Complete cartilage coverage after 2 months in vitro	Zopf et al. (2015)

Stereolithography	GelMA/PEGDA and transforming growth factor beta 1 (TGF- β 1)	UV light (photoinitiator 2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone)	Human bone marrow MSCs	In vitro	Articular cartilage	- Limiting cell proliferation with the increase in PEGDA concentration - Increase of collagen type II, aggrecan, SOX-9, and SOX-2 gene expressions with the presence of TGF-β1 nanospheres	Zhu et al. (2018)
Stereolithography	GelMA/HAMA	Blue light (385–405 nm) (photoinitiator: lithium phenyl-2,4,6-trimethylbenzoylphosphine)	Porcine chondrocyte cells	In vitro	Articular cartilage	- Collagen type II and aggrecan gene expressions after 14 days - Increase in chondrogenic differentiation with the presence of GelMA - Increase in cell viability with the presence of HAMA	Lam et al. (2019)
Stereolithography	Decellularized porcine cartilage extracellular matrix/GelMA/exosome	Visible light (photoinitiator: lithium phenyl-2,4,6-trimethylbenzoylphosphine (LAP))	Bone marrow-derived mesenchymal stem cells (BMSCs)	In vitro and in vivo (rat and rabbit models)	Articular cartilage	Induction of osteochondral defect repair in rabbit models after 6 and 12 weeks	Chen et al. (2019)
Stereolithography	Chitosan/PEGDA	UV light (photo initiator: Igacure 819)	Human mesenchymal stem cells	In vitro	Articular cartilage	- Cell viability of more than 90% after 7 days - Improvement of cell attachment to the	Sun et al. (2015)

(continued)

Table 13.2 (continued)

3D printing method	Biomaterial	Cross-linking method	Cell type	Experimental phase	Cartilage type	Regeneration outcome	Ref
Stereolithography	Poly-D,L-lactic acid/polyethylene glycol/poly-D,L-lactic acid (PDLLA-PEG) and HA	Visible light (photoinitiator: LAP)	Human adipose-derived stem cells (hASCs)	In vitro	Articular cartilage	surface of the scaffold with the presence of chitosan – Increase in cell viability from 65% to 77% after 28 days with the presence of TGF- β 3 in cell media – Increase in collagen type II, aggrecan, and SOX 9 gene expressions with the addition of TGF- β 3 in cell media	Nimbalkar (2017)
Stereolithography	PCL/Alginate/TGF β	CaCl ₂ solution	Human nasal septal cartilage chondrocyte cells	In vitro and in vivo (mice models)	Articular cartilage	– Improvement in cartilage regeneration in mice models after a month with the presence of chondrocytes and TGF β	Kundu et al. (2013)
Stereolithography	Poly-(trimethylene carbonate) (PTMC)	Blue light (400–550 nm)	Bovine chondrocyte cells	In vitro	Articular cartilage	– Adherence and proliferation of chondrocytes on the scaffold surface after 3 and 6 weeks – Expression of sulfated GAG and fibrillar collagen after 6 weeks	Schuller-Ravoo et al. (2013)

In situ	Alginate/PEGDA	UV light (Ilgacure 2959) and CaCl_2 solution	N/A	Ex vivo (rabbit models)	Articular cartilage	Cartilage-like repair in rabbit ex vivo models	Li et al. (2017b)
In situ-stereolithography	PEG	UV light (Ilgacure 2959)	Bovine chondrocyte cells	Ex vivo (pig model)	Articular cartilage	Expression of sulfated GAG and aggrecan 3 weeks post implantation in porcine knee models	Aisenbrey et al. (2018)
In situ	Norbomene-modified HA (NorHA)	Visible light (photoinitiator: LAP)	Bovine mesenchymal stem cells	In vitro	Hyaline cartilage	- Expression of collagen type II, aggrecan, and SOX 9 after 56 days - Higher expression of collagen type II comparing to collagen type I (a validation of hyaline cartilage regeneration over fibrocartilage regeneration)	Galarraga et al. (2019b)
In situ-extrusion-based	GelMA/HAMA	UV light (photo initiator: 2,2'-Azobis[2-methyl-N-(2 hydroxyethyl) propionamide] (VA086), LAP, and Igacure 2959	Allogenic adipose-derived MSCs	In vitro	Hyaline cartilage	- Decrease in cell viability by 30% after 7 days with mono-axial in situ bioprinting - Cytotoxicity of LAP photo activation	Duchi et al. (2017, 2020)

(continued)

Table 13.2 (continued)

3D printing method	Biomaterial	Cross-linking method	Cell type	Experimental phase	Cartilage type	Regeneration outcome	Ref
In situ-extrusion-based	GelMA/HAMA	LED light (photoinitiator: VA086)	Allogenic adipose-derived MSCs	In vitro and in vivo (sheep models)	Hyaline cartilage	– Regeneration of hyaline like tissue regeneration with in situ formed scaffold comparing to prefabricated scaffold implantation in sheep models after 8 weeks	Di Bella et al. (2018b)

samples containing human articular chondrocytes. It was stated that cartilage matrix-related gene expressions in the cell-laden 3D bioprinted hydrogels into the OC plug were significantly higher after 2 and 4 weeks. The full integration of 3D bioprinted hydrogel into the OC plug was also visualized. Hence, it was concluded that besides bioink properties, substrate has undeniable impact on the regeneration results (Cui et al. 2012). In another study, the impact of simultaneous photo-cross-linking during the 3D bioprinting process was compared with photo-cross-linking a 3D bioprinted scaffold in the postprocessing step. Various concentrations of alginate, human adipose-derived mesenchymal stem cells (hASCs), and murine fibroblasts (NIH-3T3-GFP) were 3D bioprinted using an inkjet-based 3D bioprinter. CaCl_2 cross-linking and UV light photopolymerization were applied both during and after the printing process to investigate the impact on cell distribution and viability. The lowest concentrations of alginate (0.5 and 1 w/v %) were reported to get poor resolution hydrogels and unacceptable mechanical properties. It was observed that UV cross-linking after the 3D bioprinting process decreased cell viability by 20% after 1 day of cultivation (Raddatz et al. 2018). To increase the mechanical strength of the scaffold, electrospinning technique was used to form the top and bottom layers of a 3D printed scaffold. A layer-by-layer scaffold based on the PCL, collagen, and fibrin was fabricated encapsulating chondrocytes cells to evaluate cartilage regeneration of the construct. Thrombin was added to cross-link fibrin in the 3D bioprinted samples before the addition of the last electrospun layer. It was reported that the proposed scaffold had about 82% cell viability after a week. It was observed that collagen type II and GAG depositions were low after 2 weeks of in vitro cultivation but they increased after 4 weeks. However, it was discussed that even after 4 weeks, cells encapsulated in the center of the scaffold were less active than cells distributed in the corner of the construct. This was concluded to be due to the lack of nutrient diffusion to the center of the scaffold. Nevertheless, in vivo analyses after 8 weeks were reported to show dense and well-organized collagen deposition. Therefore, despite of in vitro limitation, it was claimed that cells encapsulated in the proposed layer-by-layer scaffold structure showed a large amount of collagen type II and GAG deposition in the mice models (Xu et al. 2013).

13.3.1.2 Extrusion-Based 3D Printers

In extrusion-based 3D printers, a piston or pneumatic force will be applied to bioink solution to deposit it onto the substrate (Huang et al. 2021b). Kosik-Kozio et al. investigated the impact of ceramic microparticles on GelMA/alginate-based 3D printing scaffold for calcified cartilage tissue engineering. A coaxial extrusion-based 3D printer was used to fabricate the construct having GelMA/alginate and β -tricalcium phosphate (TCP) in the shell and CaCl_2 in the core part of the 3D printer. After 3D bioprinting, samples were exposed to UV light for further cross-linking of the GelMA. TCP concentration was optimized according to the efficiency of the UV cross-linking of the GelMA in the bioink solution. It was observed that the increase of TCP concentration reduced photo-cross-linking density. Hence, the lowest concentration of TCP was used for in vitro evaluations. It was claimed that TCP presence did not affect the compression modulus of the 3D printed samples

whereas it reduced the storage modulus of the hydrogels. The viability of the human bone marrow mesenchymal stem cells (hBMSCs) was observed to be more than 80% after 21 days of cultivation. The depositions of aggrecan, collagen type II and X were showed to have increments in the 3D bioprinted samples after 21 days in the presence of TCP while collagen type I expression was not observed to be affected by ceramic microparticles. Expression of cartilage-related genes was observed to increase with the presence of TCP in the 3D bioprinted hydrogels. This scaffold was proposed to regenerate calcified cartilage tissue which requires further in vivo investigation to evaluate its regeneration potentials (Kosik-Kozio et al. 2019). In another study, gelatin/hydroxyapatite (HAP) solution was cross-linked by both physical and enzymatic cross-linking procedure. An interwoven structure was 3D bioprinted containing human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSC) using a microextrusion-based 3D bioprinter. It was reported that HAP presence slowed down gelation kinetic and increased mechanical strength of the 3D-printed scaffold. Scaffolds containing HAP were observed to be more cytotoxic but the overall cell viability of the gelatin-HAP scaffolds was acceptable (more than 75%). It was shown that the proposed scaffold supported hUCB-MSCs growth, proliferation, and migration. It was observed that gelatin-HAP scaffolds promoted the formation of cartilage-like tissue and enhanced chondrogenic differentiation of the encapsulated cells in vitro after 21 days. For in vivo investigation, a square-shaped damage was created on the cartilage of the knee joints in pig models and animals were sacrificed after 3 and 6 months to study the regeneration potential of the 3D bioprinted gelatin-HAP scaffolds. About 70% and 100% repair was reported in the animal groups treated by using 3D bioprinted the gelatin-HAP-based structures after 3 and 6 months, respectively. The new regenerated tissue in these groups was observed to appear close to the native cartilage tissue (Huang et al. 2021a). Hybrid structures have attracted scientists' attention in cartilage tissue engineering because of their reinforced structure as well as high cytocompatibility (Mohseni et al. 2022). A hybrid structure containing beta cyclodextrin (β -CD)-modified alginate/decellularized cartilage ECM hydrogel and PCL/starch microfibers was developed as a carrier for hADMSCs. Kartogenin (KG) was loaded into β -CD-modified alginate/decellularized cartilage ECM hydrogel to induce cartilage tissue-specific marker differentiation of hADMSCs. PCL/starch microfibers were 3D printed using an extrusion-based 3D printer in order to reinforce the aforementioned hydrogel. It was observed that cell proliferation and viability did not have any significant difference between samples containing β -CD-modified alginate and hybrid structures containing unmodified alginate. On the other hand, GAG content was shown to improve remarkably in hydrogels incorporated with β -CD-modified alginate on days 14 and 21 (Mohseni et al. 2022). A hybrid structure composed of HA, alginate, and PLA was fabricated using an extrusion-based 3D printer. An interwoven disk-shaped structure was first 3D printed with PLA. Then, HA/alginate solution containing human chondrocytes was injected in between of the pore structures of the 3D printed structure. The final fabricated product was soaked into a CaCl_2 solution for ionic cross-linking. HA incorporation increased mechanical properties of the construct comparing to the scaffold containing PLA alone. This was

argued to be due to the large colloid osmotic pressure and viscoelasticity characteristics of HA. Cell viability was reported to be 85% after the 3D bioprinting process. The presence of HA also shown to improve the chondrogenic matrix production. It was reported that collagen type II and GAG expressions were increased after 4 weeks in the samples containing HA. Moreover, hyaline cartilage-specific gene expressions (SOX9, COL2A1, and ACAN) were reported to increase with the presence of HA (Antich et al. 2020). The effect of co-printed methacrylated poly [N-2-hydroxypropyl) methacrylamide mono/diacrylate] (pHPMA-lac)/poly ethylene glycol (PEG)/methacrylated hyaluronic acid (HAMA) with PCL scaffolds was investigated using an extrusion-based 3D printer on chondrocyte cell behavior by Mouser et al. Various concentrations of HAMA were studied to improve the properties of the proposed scaffold. It was reported that the increment of HAMA concentration promoted collagen type I expression which is a marker for fibrocartilage tissue. Contrarily, collagen type VI expression which is a marker for chondron formation decreased in the samples containing higher concentration of HAMA. This trend is also observed in proteoglycan IV expression which is a zonal marker in the cartilage surface. Interestingly, it was reported that different cell donor did not affect GAG content which was concluded to be due to HAMA presence in the structure of the 3d bioprinted scaffolds (Mouser et al. 2017). Moreover, it was reported that the presence of graphene oxide (GO) nanofiller in 3D bioprinted scaffold containing alginate/GelMA/chondroitin sulfate (CS) as a bioink improved the compressive elastic modulus. Cells encapsulated in the scaffolds containing both GelMA and CS were observed to have more cell-material interaction comparing to the cells encapsulated within pure alginate samples. A small amount of GO (0.1 mg/mL) was shown to have positive impact on cell proliferation while higher concentrations (1 mg/mL) were argued to form cell aggregations. It was revealed that the presence of GO in the scaffold structure did not affect cell viability. Cells encapsulated in samples composed of 0.1 mg/mL GO were reported to have high level of collagen type II, aggrecan, and SOX 9 expressions after 28 days. It was discussed that GO have chondro-inductive properties and GelMA and CS improved cells adhesion and growth (Olate-Moya et al. 2020). The combination of Poly(N-2-hydroxypropyl) methacrylamide-mono/dilactate-polyethylene glycol (pHPMALac-PEG) and methacrylated chondroitin sulfate (CSMA) was studied as a proper bioink for cartilage regeneration. It was reported that this combination increased rheological properties of the scaffold and caused a shear-thinning behavior. The percentage of ATDC5 cells viability in various days after cultivation was shown to be as day1 > day7 > day4. Cytocompatibility of the cells encapsulated within 3D bioprinted pHPMALac-PEG/CSMA scaffold was observed to be lower than the cells encapsulated in pure pHPMALac-PEG and pure CSMA hydrogels on days 4 and 7 (Abbadessa et al. 2016). It was argued that this set up is difficult to apply clinically (Liu et al. 2022). Primary fibrochondrocytes-laden 3D bioprinted hydrogel composed of various concentrations of collagen was reported to be applied for fibrocartilage regeneration. It was shown that the mechanical properties of the construct increased with increasing the collagen concentration. This was also observed in shape fidelity assessment.

However, it was reported that collagen concentrations higher than 20 mg/mL caused poor shape fidelity and collagen was deposited as a gel. It was observed that cell viability remained higher than 90% in all the samples after 10 days (Rhee et al. 2016). Furthermore, the increase in fibrinogen concentration in gellan gum and methacrylated silk fibroin (Si-MA) composition was reported not to be proportional to the increase in mechanical properties. The mechanical strength was enhanced in a hybrid structure of (Si-MA) and gellan gum/fibrinogen (GG/FB). Collagen/GAG contents and ECM deposition similar to native fibrocartilage tissue were detected after 10 weeks of implantation in mice models (Costa et al. 2020).

13.3.1.3 Laser-Based 3D Printers

In laser-based 3D printers, a laser beam will be applied to an absorbent substrate on which the bioink is spread to transfer geometry to the bioink solution (Huang et al. 2021b). A multilayer PCL-based 3D bioprinted scaffolds were designed with the various concentrations of hydroxyapatite nanoparticles (HANP) in different layers to investigate osteochondral repair in rabbit models. Selective laser sintering (SLS) technique was used to fabricate a seven-layer scaffold with the continuous HANP gradient (HA content continuously increased through top to bottom layers). The addition of HA was shown to decrease the mechanical properties of the scaffold which was argued to be due to the poor integration between HA nanoparticles and PCL. Rat MSCs viability and adhesion were confirmed in the multilayer 3D-printed sample. It was observed that HANP improved cell osteogenic differentiation. To study scaffold regeneration potential in vivo, the 3D-printed structures were implanted into defected rabbit knees for 6 months. It was reported that defects filled with the multilayer scaffold showed full cartilage-like tissue regeneration, while animal models treated with pure PCL scaffolds had a small amount of cartilage-like tissue with small cavities on the defected site. It was shown that with increasing implantation period from 6 to 12 weeks, tissue regeneration was enhanced in both groups. However, an obvious border was detected in the animal models treated with pure PCL structures. Immunohistochemical staining was applied to visualize aggrecan, collagen type II, collagen X, osteocalcin, and collagen type I expressions after 6 and 12 weeks. A large amount of collagen II expressions after 12 weeks were reported to validate hyaline-like cartilage tissue formation. Collagen X was observed after 6 weeks as a hypertrophic phenotype for tissue calcification but disappeared after 12 weeks, which is another sign of hyaline cartilage tissue regeneration. Osteocalcin and collagen type I expressions were also observed in the multilayer scaffold which indicated the subchondral bone regeneration. Therefore, it was concluded that this multilayer scaffold could be a great candidate for both subchondral bone repair and articular cartilage regenerations (Du et al. 2017). Moreover, laser sintering system was used to 3D bioprint ear and nose cartilage tissue using PCL as a bioink. Chondrocytes isolated from porcine articular cartilage were encapsulated in 3D bioprinted scaffolds with precise geometry. It was reported that full cartilage tissue regeneration was not observed after 2 months in vitro. The in vivo investigation was claimed to be ongoing (Zopf et al. 2015).

13.3.1.4 Stereolithography 3D Printers

In stereolithography, a source of light will be applied to a photo-cross-linkable bioink solution and the geometry will be formed in a layer-by-layer process (Huang et al. 2021b). A stereolithography-based 3D printing was used to encapsulate MSCs and TGF- β 1 nanospheres into GelMA/PEGDA scaffold for articular cartilage regeneration. The addition of PEGDA to GelMA was observed to increase mechanical properties of the scaffold. This was argued to be one of the main reasons for embedding PEGDA in 3D bioprinted structures. It argued to improve shape fidelity and 3D bioprinting resolution. However, it was shown that the increment of PEGDA concentration caused a compact structure which limited cell growth and proliferation. It was reported that the presence of TGF- β 1 nanospheres augmented collagen type II, aggrecan, SOX-9, and SOX-2 gene expressions within 3 weeks. Although further in vivo investigations are required to study the regeneration potential of the proposed bioink, it was observed that the combination of TGF- β 1, GelMA, and PEGDA is in favor of chondrogenic differentiation of MSCs (Zhu et al. 2018). It was argued that in the 3D bioprinted scaffold composed of GelMA and HAMA, HAMA had more impact on improving cell cytocompatibility and GelMA enhanced chondrogenesis differentiation in porcine chondrocytes (Lam et al. 2019). The addition of decellularized porcine cartilage extracellular matrix (D-ECM) to GelMA was observed to increase surface roughness of the 3D-printed sample. Moreover, a control release of exosome was observed for 7 days in GelMA/D-ECM hydrogel. The presence of D-ECM had positive impact on the migration of BMSCs. No inflammatory response was observed after the implantation of 3D bioprinted samples in rat models for 3 weeks and the scaffold was completely degraded within this period. Cartilage tissue and osteochondral regeneration were detected in rabbit models after 6 and 12 weeks (Chen et al. 2019). In a study by Sun A.X et al., various 3D shapes such as conical, cubic, and cylindrical geometries were 3D bioprinted using a visible light projection stereolithography-based technique. Methacrylated poly-D,L-lactic acid/polyethylene glycol/poly-D,L-lactic acid (PDLLA-PEG), and hyaluronic acid were used as a bioink for human adipose-derived stem cells (hASCs) delivery. Cell viability was reported to be 81% immediately after the 3D bioprinting process and it decreased to 65% after 28 days. It was pointed out that by the addition of TGF- β 3 to the cell media, cell viability was 77% after 28 days. Chondrogenic differentiation was studied with and without the presence of TGF- β 3 in cell media. It was discussed that SOX 9, collagen type II, and aggrecan gene expressions were higher in the samples containing TGF- β 3 in cell media while gene expression of Runx2 which is an osteogenesis marker was higher in the samples without TGF- β 3 after 28 days of cultivation. Therefore, the biomaterial alone was not sufficient to induce remarkable chondrogenic gene expression in hASCs. The presence of TGF- β 3 in cell media also reported to cause collagen type II protein expression, and significantly improve GAG and proteoglycan contents after 28 days (Sun et al. 2015). An increase in PEGDA concentration in a mixture of PEGDA and chitosan was observed to decrease hMSCs viability and attachment. However, it was reported that cell viability was more than 90% for all the cases over time. This was argued to be due to the ionic interactions between cationic chitosan

and anionic GAG (Nimbalkar 2017). A single layer of PCL-alginate was reported to have more than 95% human nasal septal cartilage chondrocyte cells viability while the cytocompatibility decreased by the addition of more layers. This discussed to be upon staking more layers of PCL. The addition of TGF β to the construct was reported to improve DNA content and GAG expression after 4 weeks of cultivation both in vitro and in vivo. Collagen II fibers were also visualized in animal models treated by the proposed composition. The presence of both chondrocytes and TGF β were argued to improve cartilage regeneration in mice models (Kundu et al. 2013). A cylindrical 3D porous scaffold was designed using Poly-(trimethylene carbonate) (PTMC) for articular cartilage regeneration. Bovine chondrocytes were seeded on scaffold surface post printing. It was observed that cells adhered to the scaffold structure after 3 weeks and full cell confluency were reported after 6 weeks. The production of sulfated GAGs and fibrillar collagen was visualized after 6 weeks. It was discussed that improving hydrophilicity of the scaffold might enhance chondrogenesis differentiation (Schuller-Ravoo et al. 2013).

13.3.1.5 In Situ 3D Printer

In situ 3D bioprinting is a promising approach to eliminate postprocessing step (Galarraga et al. 2019b). In situ 3D bioprinting has attracted scientists' attention because it provides tissue regeneration in a minimum invasive approach which also avoids damaging the surrounding cartilage tissue. It was argued that alginate hydrogel is a good candidate for treating osteochondral defect (Fig. 13.3a). It was observed that exposing alginate hydrogel to UV light turned its color from yellow to white and made it more like a native cartilage tissue (Fig. 13.3b–f) (Li et al. 2017b). PEG was used as a hydrophilic bioink for in situ stereolithography 3D bioprinting to improve scaffold-tissue interaction. It was reported that the 3D bioprinted scaffold remained in place after 3 weeks even under mechanical loads. Sulfated GAG and aggrecan expressions were also observed in the regions adjacent to the treated spot in porcine knee ex vivo models (Aisenbrey et al. 2018). In another study, norbornene-modified HA (NorHA) was used a nonviscous bioink for in situ delivery of MSCs. It was shown that high 3D bioprinting flow rate resulted in low printing resolution. Cell viability was reported to be more than 85% after a week of cultivation. Cell distribution was argued to be homogenous through the 3D printed structure. The expressions of chondrogenic markers such as collagen type II, aggrecan, and SOX9 were observed after 56 days of incubation. Sulfated GAG and collagen contents were also claimed to increase after 56 days which was discussed to be a sign of neocartilage formation. Collagen type I expression was reported to be less than collagen type II which was implied as a proof for the hyaline cartilage regeneration rather than a fibrocartilage formation (Galarraga et al. 2019b). The impact of three various photoinitiators such as (2,2'-Azobis [2-methyl-N-(2 hydroxyethyl) propionamide] (VA086), lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), and Igacure 2959 on scaffold properties was studied using GelMA/HAMA as the bioink. It was reported that increasing UV exposure time improved mechanical strength of the in situ 3D bioprinted samples. It was observed that using VA086 as a photoinitiator resulted in the lowest compressive modulus while using LAP

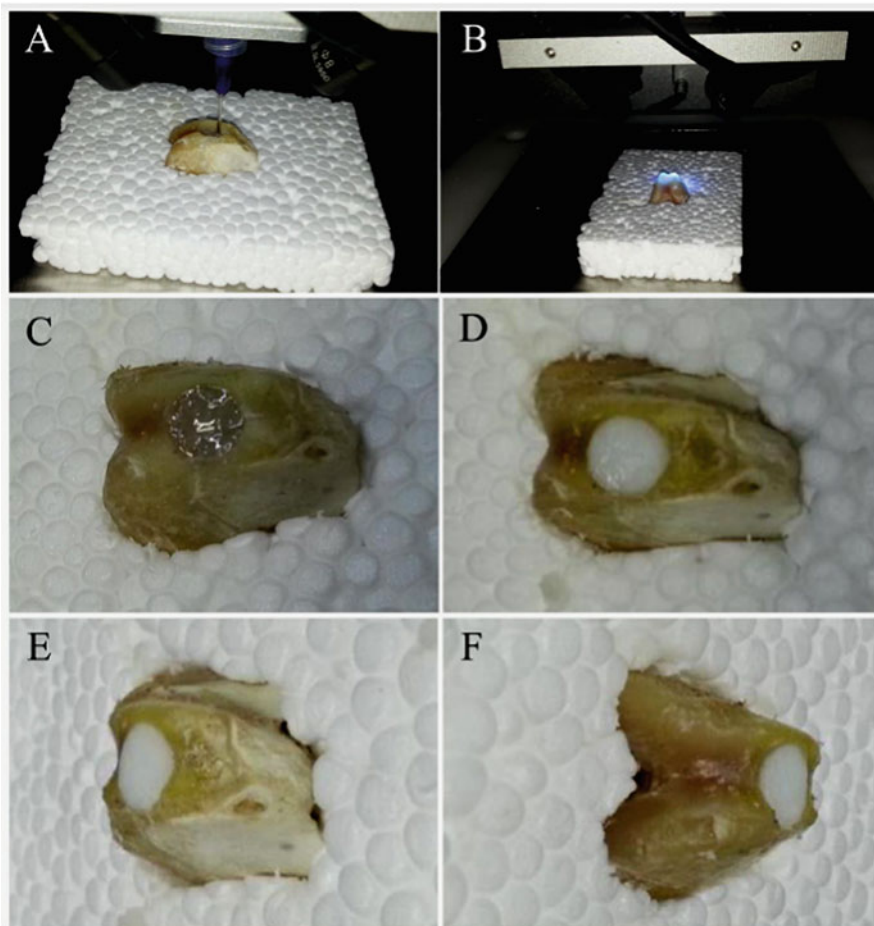


Fig. 13.3 3D printing of cartilage patches for treating cartilage defects. (a) In situ 3D bioprinting with alginate hydrogel to repair an osteochondral defect. (b) Exposure of the printed sample to UV light. (c) Color of the alginate hydrogel before photopolymerization. (d–f) Changing of the alginate hydrogel after being exposing to UV light. Adapted from (Li et al. 2017b) licensed under creative commons license

provided the highest compressive modulus even at shorter UV exposure time. It was observed that exposing cells to the UV light in the absence of LAP was not toxic to the cells; however, the presence of LAP caused decrease in cell viability after exposing samples to UV light for in situ printing and this was not proportional to LAP concentration. It was argued that generated free radicals by LAP-photo activation was the main reason behind cytotoxicity of the samples after 1 day. Nevertheless, it was demonstrated that by incubating cells within GelMA/HAMA scaffold for 7 days, the cytotoxic effect of LAP was shielded by the biomaterials' biocompatibility. It was also discussed that mono-axial in situ printing reduced cell viability by

30% comparing to the samples fabricated using co-axial in situ printing after 7 days. Moreover, increase in cell density was shown to improve cell proliferation and growth in co-axial 3D-printed samples (Duchi et al. 2017, 2020). This bioink was utilized to treat a full thickness cartilage defect in a single session surgery in sheep models for further investigation. The biopolymers and photoinitiators were feed into the shell and MSCs mixed with the biopolymers were inserted into the core in a shell/core 3D bioprinted setup. No inflammatory and immune system response was observed 8 weeks postoperation period. Interestingly, it was reported that newly formed tissue was mostly like native hyaline cartilage in animals treated with in situ 3D bioprinted scaffolds after 8 weeks comparing to animals received prefabricated 3D bioprinted scaffold containing same biopolymers (Di Bella et al. 2018b).

13.3.2 Scaffold-Free 3D Printing

To eliminate exogenous materials regarding the cytotoxicity of degradation byproducts and inflammatory responses, scaffold-free methods were developed for cell delivery (Zhang et al. 2009). In this approach, the living cells are printed directly to the defect area which is known for mimicking the embryonic growth (Gopinathan and Noh 2018). List of scaffold-free 3D printing studies in cartilage tissue engineering are summarized in Table 13.3.

Cartilage tissue strands were formed in alginate-based capsules using primary chondrocytes (Fig. 13.4a, b). Co-axial extrusion-based 3D printer was utilized to produce microtubular alginate capsules. Cell viability was reported to increase from 75% at day 1 to 87% on day 7. Sulfated GAG, collagen type II, aggrecan, and SOX 9 expressions were observed to be remarkable after 2 weeks comparing to the control group (native bovine cartilage samples). Tissue strands were implanted into a bovine osteochondral model for 4 weeks. It was shown that implanted tissue was adhered to the defect; however, the full integration was not observed (Yu et al. 2016). MSCs spheroids were 3D bioprinted to fabricate a 3D tubular structure as an artificial trachea. It was reported that tensile strength of the 3D-printed artificial trachea increased over the 5 weeks period. It was shown that GAG content and the expression of collagen type II in rat models received artificial trachea were higher than animals treated with trachea grafts. Moreover, the appearance of microvessels was observed after 8 days in rat models received scaffold-free 3D-printed MSCs spheroids (Taniguchi et al. 2018). MSCs pellets were 3D printed into various geometries using a laser-based 3D printer. Sulfated GAG, Collagen type II, and aggrecan expressions were observed after 21 days of cultivation in both 3D-printed and nonprinted MSCs with no significant difference. It was confirmed that 3D printing process did not have negative impact on cytocompatibility and cell proliferation (Gruene et al. 2011).

Table 13.3 Scaffold-free 3D-printed samples for cartilage regeneration

3D printing method	Cell type	Experimental level	Cartilage type	Regeneration outcome	Ref
Scaffold-free-extrusion-based	Bovine chondrocyte cells	In vitro and ex vivo (bovine models)	Articular cartilage	– Cell viability of 87% after a week of cultivation – Adherence of produced tissue strands to bovine osteochondral model after 4 weeks	Yu et al. (2016)
Scaffold-free	Rat mesenchymal stem cells	In vitro and in vivo (rat model)	Hyaline cartilage	– Higher GAG content and collagen type II in animals treated with scaffold-free MSCs spheroids comparing to the rat models received trachea graft	Taniguchi et al. (2018)
Scaffold-free-laser based	Porcine bone marrow stem cells	In vitro	Articular cartilage	– Expression of sulfated GAG, collagen type II, and aggrecan after 21 days of cultivation	Gruene et al. (2011)

13.4 Conclusion and Future Aspects

3D printing is shown to be a promising approach for fast and long-term cartilage tissue regeneration. 3D printing techniques provide a vast variety of scaffold-based and scaffold-free approaches for cartilage tissue treatment. It also provides the opportunity to repair a cartilage defect in a minimally invasive manner using in situ 3D printing methods. Furthermore, in scaffold-based 3D printing, physical, chemical, mechanical, and biological properties of the construct can be adjusted to stimulate encapsulated cells to produce a cartilage tissue-like microenvironment. The main goal of any tissue engineering approaches including 3D printing is to replace the donor tissue transplantation with the artificial bioengineered scaffold (Mahdavi and Mashayekhan 2022; Mahdavi et al. 2020a; Zhang et al. 2017). The ultimate step to assess the potential of the proposed method/idea for cartilage tissue repair is to advance to the clinical tests after the in vitro/ex vivo and in vivo investigations. Biocompatibility and shape fidelity are two of the most common challenges in 3D printing that should be overcome to proceed to the clinical trials. Moreover, as tissue engineering and regenerative medicine approaches progress toward the clinical experiments, the geometry and mechanical strength of the

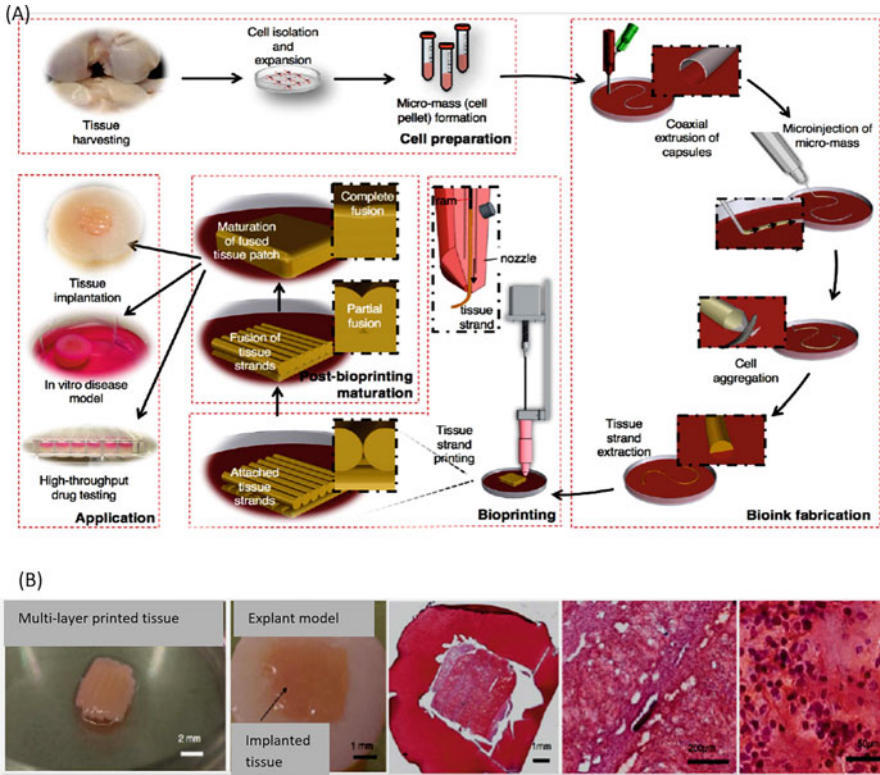


Fig. 13.4 (a) 3D printing of scaffold-free tissue strands. (b) Implantation of 3D-printed tissue strands into osteochondral defect and Safranin-O/Fast Green histology examination at different magnifications. Adapted from (Yu et al. 2016) licensed under creative commons license

bioengineered construct should mimic the native cartilage tissue as closely as possible (McGivern et al. 2021). In this case, the emergence of 4D bioprinting approaches has revolutionized the potential of 3D bioprinting techniques for cartilage tissue engineering. In 4D bioprinting, the alteration of physical, chemical, mechanical, and biological properties happens with applying external stimulation in a time-dependent manner (Yang et al. 2022a; McGivern et al. 2021). This can be considered as a remarkable progress in cartilage tissue healing because it gives an adaptable construct to inexorable environmental factors mimicking human body mechanism. In general, 4D printing can provide three main characteristics of self-assembling, multifunctional, and self-repairing (Yang et al. 2022a). Few studies have applied 4D printing techniques for cartilage tissue regeneration and introduced it as a great transformation of the 3D bioprinting (Betsch et al. 2018; Qasim et al. 2019). Advances in machine learning and artificial intelligence are facilitating the rate of growth of this technology (McGivern et al. 2021). So far, light-assisted and extrusion-based 3D bioprinters have been upgraded to be used in 4D bioprinting approaches (Yang et al. 2022a). The lack of machine learning and artificial

intelligence trained users and the rapid speed of the appearance of new algorithms are some of the challenges slowing down the rate of development in this method (McGivern et al. 2021). Nevertheless, 3D printing approaches are becoming an essential tool in cartilage tissue engineering and they are expected to change the methods of treatment in near future.

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Cell Therapy as a Novel Therapeutic Approach for Cartilage Diseases

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Zachariah Gene Wing Ow, Derrick Guo, Heng An Lin,
Merng Koon Wong, and Keng Lin Wong

Abstract

Autologous Chondrocyte Implantation (ACI) has long been considered the gold standard for the repair of full-thickness articular cartilage defects, with evidence of up to 20 postoperative years supporting its use. However, certain inherent limitations with the ACI procedure have sparked a gradual shift in interest to explore the development of Mesenchymal Stem Cells (MSCs) for the treatment of such cartilage lesions. This chapter explores the role of MSCs as a novel, cellular-based therapy for articular cartilage lesions. With the increasing amount of high-quality evidence emerging in support of the efficacy of MSCs for the treatment of cartilage lesions, the novelty status of such therapies is challenged by the potential for MSCs to hold the key to the future of personalised medicine in the area of cartilage regeneration.

Z. G. W. Ow

Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

D. Guo

Department of Orthopaedic Surgery, Woodlands Health Campus, Singapore, Singapore

Department of Orthopaedic Surgery, Sengkang General Hospital, Singapore, Singapore

H. A. Lin · M. K. Wong

Department of Orthopaedic Surgery, Sengkang General Hospital, Singapore, Singapore

K. L. Wong (✉)

Department of Orthopaedic Surgery, Sengkang General Hospital, Singapore, Singapore

Musculoskeletal Sciences Academic Clinical Programme, Duke–NUS Graduate Medical School, Singapore, Singapore

e-mail: francis.wong.k.l@singhealth.com.sg; gmswke@nus.edu.sg

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M. Baghaban Eslaminejad, S. Hosseini (eds.), *Cartilage: From Biology to Biofabrication*, https://doi.org/10.1007/978-981-99-2452-3_14

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Keywords

Articular cartilage · Cartilage regeneration · Cell-based therapy · Mesenchymal stem cells · Surgery

14.1 Introduction

Long considered the gold standard of cell-based cartilage repair, Autologous Chondrocyte Implantation (ACI) and Matrix-Induced ACI (MACI) have displayed excellent safety profiles and clinical efficacy for up to 20 postoperative years (Ogura et al. 2017; Peterson et al. 2010). However, ACI procedures are inherently limited by the need for two separate surgeries, issues with dedifferentiation of implanted chondrocytes, and donor site morbidity (Matricali et al. 2010; Duan et al. 2015). Hence, interest has grown in the search for alternative cellular sources that circumvent these limitations, with Mesenchymal Stem Cell (MSC)-based therapies being one of the most explored options in recent years. MSC-based therapies center around two components of intervention, namely, harvesting and culturing exogenous MSCs, followed by the subsequent introduction of MSCs into the site of diseased cartilage. Various tissue sources can be considered for MSC harvesting, such as the bone marrow, adipose tissue, human umbilical cord blood, and synovial membranes, with the harvested tissue being subjected to controlled, sterile culture environments in order to expand the MSC colony size prior to implantation. Currently, MSC-based therapies are being investigated for use in both isolated articular cartilage defects, as well as cartilage erosion secondary to osteoarthritis (OA), with promising preliminary results displayed in the treatment of both disease states (Tan et al. 2021; Teo et al. 2019). Whilst in theory, MSC-based therapies can be applied to most articular surfaces, an overwhelming proportion of resources have been dedicated to the treatment of cartilage lesions of the knee joint, and hence the strongest evidence surrounding the use of MSCs for cartilage regeneration center around articular lesions of femoral condyles and patellofemoral joint. A small number of studies have investigated the use of MSCs for cartilage regeneration in femoral head, talar, and capitellar lesions; however, the quality of evidence remains weak, with limited conclusions being able to be drawn from such studies. As such, this chapter primarily discusses the use of MSC therapy in the treatment of cartilage diseases of the knee joint.

MSCs promote cartilage regeneration through various diverse mechanisms. Classically, the cell engraftment and chondrogenic differentiation of implanted or injected MSCs to repopulate sites of cartilage defects was the main theory by which MSCs promoted cartilage regeneration (Iso et al. 2007). Recently, the paradigm has shifted toward accepting the theory that the main mechanism of cartilage regeneration is the paracrine signaling of preexisting native chondrocytes by the implanted MSCs in order to stimulate cellular proliferation and maintenance of a sufficient volume of newly differentiated chondrocytes, and secretion of immunomodulatory cytokines and extracellular vesicles upon exposure to the sites of cartilage damage (Zha et al. 2021; da

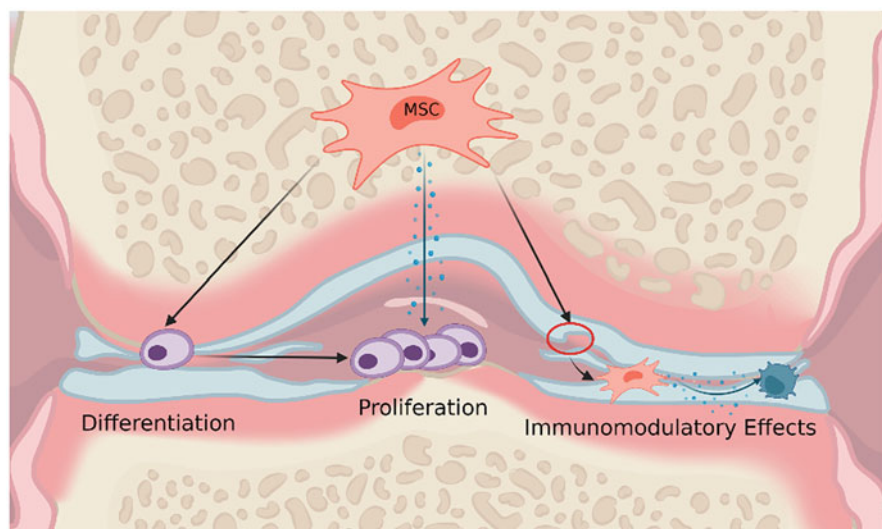


Fig. 14.1 Local effects of implanted/injected mesenchymal stem cells, including differentiation to target cell type, stimulation of cell proliferation, and immunomodulatory effects

Silva Meirelles et al. 2008; Lai et al. 2012). However, despite the preliminary evidence supporting the safety and efficacy of MSC-based therapies, there is a paucity of studies displaying the long-term safety and efficacy profile of MSCs. This, coupled with the high cost of utilizing MSCs as a treatment modality, mean that currently, MSCs still remain a novel therapeutic approach in cartilage regeneration. This chapter serves to summarize the commonly utilized cellular sources for MSC harvesting, review the techniques of MSC introduction to diseased cartilage as well as the role for adjunctive procedures, and discuss the rehabilitation process for patients treated with MSCs for cartilage lesions (Fig. 14.1).

14.2 Mechanism of Cartilage Regeneration

The articular surfaces of joints are covered with hyaline cartilage, which comprise chondrocytes as the sole cell type, and the extracellular matrix (ECM). The components of ECM are that of water (65–80% by weight), type II collagen (10–20% by weight), proteoglycans (10–15% by weight), and minimal amounts of glycoproteins (Buckwalter and Mankin 1998). Despite occupying less than 5% of the volume of articular cartilage, chondrocytes are responsible for the maintenance of the ECM, the substance that constitutes the majority of the cartilage volume. In normal physiologic states, chondrocytes are in a quiescent cellular state, with very little turnover of the ECM apart from maintaining normal composition of the ECM. This results in the characteristic smooth articular surface seen in hyaline cartilage lining joints, which allows for efficient, low-friction movements at the articular

surfaces. However, in disease states such as OA and chondral lesions, chondrocyte activity is upregulated, leading to increased cellular proliferation and production of matrix proteins and matrix-degrading enzymes, which contribute to a pro-inflammatory environment—one that is not optimal for physiologic cartilage function (Goldring and Marcu 2009). This pathologic cascade is a form of injury response to the increased mechanical stresses along the articular surfaces, and results in a loss of balance between the anabolism and catabolism of the articular cartilage matrix, leading to overall chondrocyte apoptosis and progressive erosion of the ECM (Goldring and Marcu 2009).

Hence, the manner in which MSC-based therapies seek to regenerate cartilage is through that of direct replacement and upregulation of chondrocyte proliferation, paracrine stimulation of chondrocytes to increase ECM deposition of collagen and proteoglycans, as well as immunomodulation in order to reduce the inflammatory response and return the cartilage environment to a homeostatic physiological state.

14.3 Cellular Sources

Bone marrow was the first potential tissue source of multipotent stem cells to be identified and utilized for cartilage regeneration procedures (Wakitani et al. 1994). Subsequently, other tissues such as adipose, synovium, and human umbilical cord blood, were identified to be alternative sources of viable MSCs (Han et al. 2019). All of these tissue sources yield MSCs that are capable of multidirectional differentiation, a crucial hallmark of MSCs utilized for cartilage regeneration. However, this variety of tissue sources contributes to significant heterogeneity amongst MSCs used in cartilage repair, with each source producing MSCs with varying degrees of chondrogenic differentiation tendency, proliferation capacity, and immunomodulatory ability (Zha et al. 2021).

14.3.1 Bone Marrow-Derived Mesenchymal Stem Cells (BD-MSCs)

BD-MSCs are one of the most studied and utilized cell sources for cartilage repair. BD-MSCs are sourced from bone marrow aspirate, with the iliac crest being the most commonly utilized site of aspiration (Chahla et al. 2017). Whilst other sites such as the vertebral bodies, distal femur, and proximal tibia can also be considered for bone marrow aspiration, the chondrogenic potential of harvested BD-MSCs is potentially lower than that of BD-MSCs harvested from the iliac crest (Neckař et al. 2020; Herrmann et al. 2019). Proliferation rates of BD-MSCs are comparatively lower than that of Adipose-Derived MSCs (AD-MSCs), Synovial-Derived MSCs (SD-MSCs), and human Umbilical Cord Blood-Derived MSCs (hUCB-MSCs) (Nakao et al. 2019); however, this can potentially be improved by culturing BD-MSCs in a hypoxic environment (Bornes et al. 2018). Cultured BD-MSCs display good expression of anti-inflammatory and immune regulatory cytokines that are essential for sustained survival of implanted MSCs, such as Hepatocyte Growth Factor (HGF),

Transforming Growth Factor (TGF)- β_1 , Prostaglandin (PG) E_2 , and Interleukins (IL) 6 and 10.

Currently, good evidence exists to support the use of BD-MSCs for cartilage regeneration procedures in both osteoarthritis-related cartilage erosion, as well as focal nonosteoarthritic cartilage defects. BD-MSCs have been used successfully both by means of intra-articular injection into osteoarthritic joints (Wong et al. 2013), as well as via direct implantation in the form of BD-MSC laden acellular scaffolds (Gobbi and Whyte 2016), displaying superiority to control group comparisons in terms of mid- to long-term clinical outcomes.

14.3.2 Adipose-Derived Mesenchymal Stem Cells (AD-MSCs)

Due to a relative abundance of adipose tissue sources, ease of harvesting, and good proliferation potential, AD-MSCs are becoming an increasingly utilized source of MSCs for cartilage regeneration. Whilst most commonly harvested from subcutaneous fat through simple, minimally invasive liposuction techniques, adipose tissue from the infrapatellar fat pad may be harvested arthroscopically as an alternative source, with the potential for harvesting to be performed during first-look or diagnostic arthroscopy of the diseased cartilage (Dragoo and Chang 2017). However a limitation of utilizing AD-MSCs in cartilage regeneration is that of an inferior chondrogenic differentiation potential as compared to BD-MSCs or SD-MSCs (Zha et al. 2021). Additionally, whilst AD-MSCs express good levels of HGF, their expression of other anti-inflammatory cytokines is markedly lower than that of BD-MSCs and hUCB-MSCs (Nakao et al. 2019).

Both intra-articular injections and direct implantation via acellular scaffolds of AD-MSCs have been shown to be safe and effective in the treatment of osteoarthritis and focal cartilage defects (Koh et al. 2016; Lee et al. 2019). However, good quality evidence supporting the efficacy of AD-MSC is limited to the mid-term follow-up period, with further well-designed randomized controlled trials investigating the long-term efficacy of AD-MSCs still required.

14.3.3 Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells (hUCB-MSCs)

The use of an allogenic MSC source that possesses the advantages of high proliferation, high stemness with cost effectiveness is gaining popularity amongst regenerative medicine practitioners. Whilst the collection of hUCB during delivery is routine in most developed countries, the harvesting of hUCB for MSCs is less routinely practiced. However, hUCB-MSCs do carry a wide potential for differentiation into various cell lineages, making them a versatile MSC candidate for regeneration. Additionally, hUCB-MSCs have high proliferation potential, with initial rates surpassing that of BD-MSCs and AD-MSCs (Nakao et al. 2019). Expression of anti-

inflammatory cytokines in hUCB-MSCs, whilst lower than that of BD-MSCs, is still superior to that of AD-MSCs.

Due to potential ethical concerns surrounding the acquisition of hUCB-MSCs for prospective research purposes, there is paucity of high-quality evidence to support the efficacy of hUCB-MSCs for cartilage regeneration. As of 2022, the only commercially available allogenic hUCB-MSC solution manufactured for use in cartilage regeneration is that of CARTISTEM[®] (Medi-Post, Gyeonggi-do, South Korea). This product has only received full market-approval for commercial usage in South Korea. Consequently, the east Asian nation has synthesized a vast majority of evidence surrounding the use of hUCB-MSCs. Cohort studies evaluating the efficacy of hUCB-MSC laden acellular scaffolds have displayed good mid-term clinical outcomes, superior to the outcomes observed in patients treated with concentrated bone-marrow aspirate laden scaffolds for cartilage defects (Lee et al. 2021; Yang et al. 2022). However, as CARTISTEM[®] (Medi-Post, Gyeonggi-do, South Korea) is on course to gain full FDA approval in the years following 2022, more high-quality evidence is expected to gradually emerge regarding the use of hUCB-MSCs for cartilage regeneration.

14.3.4 Synovial-Derived Mesenchymal Stem Cells (SD-MSCs)

SD-MSCs are sourced directly from the synovial membrane lining the interior surface of articular capsules of joints. Commonly harvested under direct visualization on joint arthroscopy, novel techniques of harvesting synovium through minimally invasive ultrasound-guided incisions do exist, however the efficacy and safety of the latter are still unproven (Fernandes et al. 2018; Ozeki et al. 2021). Whilst requiring the most invasive harvesting technique for source tissue, SD-MSCs possess a strong capacity for cellular proliferation and chondrocyte differentiation, superior to that of BD- and AD-MSCs (Sasaki et al. 2018; Zheng et al. 2015).

The major limitation of utilizing SD-MSCs for cartilage regeneration is the invasiveness of cell source harvesting procedures. Consequently, the evidence pool surrounding SD-MSCs for cartilage regeneration in humans is limited. SD-MSCs have been shown by one well-designed randomized controlled trial to not only be safe and effective when utilized via direct implantation to cartilage defects, but also superior to matrix-assisted ACI procedures at mid-term follow-up (Akgun et al. 2015) (Fig. 14.2).

14.4 Cellular Delivery Techniques

MSCs used for cartilage regeneration procedures are introduced to the diseased cartilage by means of either direct implantation on the site of cartilage defects, or by intra-articular injection into a diseased joint. For large, isolated cartilage defects, the consensus has often been that direct implantation was more suitable for cartilage regeneration. However, in the disease state of osteoarthritis, wherein cartilage erosion is more diffuse, the consensus is not as clear. Certain schools of thought

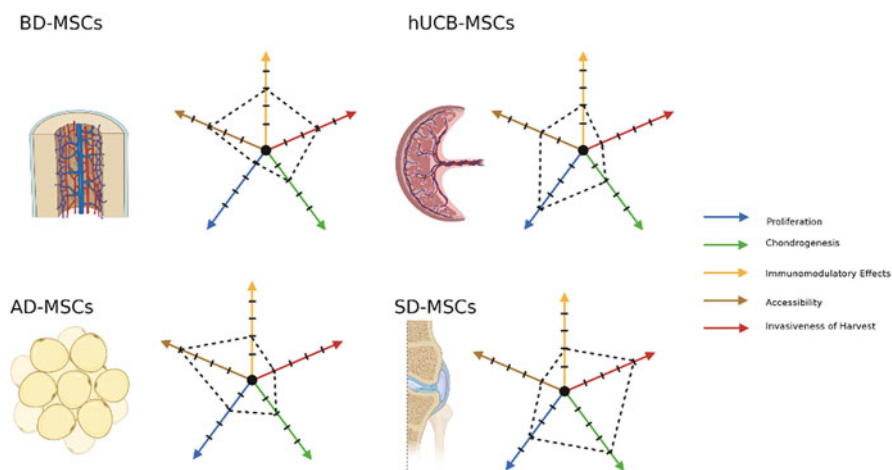


Fig. 14.2 Comparison of MSC sources in terms of their various properties

are in line with the belief that intra-articular injections are more suited to this diffuse, erosive disease state so as to saturate the joint capsule with viable MSCs, allowing for widespread cartilage regeneration. However, emerging evidence suggests that even for diffuse full-thickness cartilage lesions found in high-grade osteoarthritis, direct implantation of MSCs on acellular scaffolds yields better clinical and radiological outcomes than intra-articular injections of MSCs (Kim et al. 2015a). Nonetheless, both intra-articular injections, as well as direct implantation of MSCs are still being investigated as treatment options for cartilage regeneration.

14.4.1 Direct Implantation

MSCs implanted directly at the site of cartilage defects are typically seeded on acellular scaffolds prior to implantation, as a crucial hallmark of direct MSC implantation is ensuring the stability and proper filling of the repaired defect. Commonly used biodegradable acellular scaffolds utilized are that of nonwoven hyaluronic acid matrices such as Hyalofast[®] (Anika Therapeutics), and woven bilayer collagen I/III membranes such as ChondroGide[®] (Geistlich Pharma). These solid, layered scaffolds are cut to match the size of the cartilage defect and impregnated with the cultured MSC solution prior to implantation. Once implanted, these scaffolds containing the MSCs are secured in place by means of fibrin-based tissue glue or by suturing the matrix to the surrounding cartilage. Certain gel-based scaffolds have also been utilized for MSC delivery such as Cartifill[®] (Sewon Cellontech), an atelocollagen acellular gel scaffold, and CarGel (Smith and Nephew), which is derived from chitosan. These gel-based scaffolds are mixed

with the cultured MSC solution and fibrin-based tissue glue prior to implantation in order to increase the stability of the cartilage repair. Historically, gel-based scaffolds containing MSCs have been secured using an autologous periosteal patch that is sutured over the implanted gel to the surrounding cartilage. The periosteal patch used in this technique is typically harvested from the ipsilateral anteriomedial proximal tibia and cut to match the size of the defect; however, this technique has fallen out of favor with the advent of the solid, layered scaffolds (Haleem et al. 2010). There is no current consensus on the indications for or efficacy of each type of acellular scaffold for use with MSC implantation; however, a study examining the clinical and radiological outcomes of each scaffold type when utilized for autologous matrix-induced chondrogenesis (AMIC) procedures for cartilage regeneration showed that minimal clinically significant differences were found when comparing single-, multilayered, and gel-based acellular scaffolds (Ow et al. 2022).

The surgical procedure for preparing the site of chondral defect typically utilizes shavers and curettes, and involves debriding diseased, calcified cartilage to the level of subchondral bone, and shaving the peripheries of the lesion to ensure stable vertical margins (Heng et al. 2021). This may be performed either via arthroscopic or open approaches, depending on the size and location of the cartilage lesion. Microfracture is a routine practice in ACI and AMIC procedures, however, the practice is rarely performed in cartilage repair involving MSC implantation, as the purpose of microfracture is to stimulate the migration of MSC-rich bone marrow into the site of the cartilage defect, a phenomenon that is no longer necessary when directly implanting cultured MSCs into the site of the lesion.

14.4.2 Injection

Compared to the technique of directly implanting an acellular scaffold laden with cultured MSCs onto a cartilage defect, the process of injecting cultured MSCs into the articular space containing cartilage defects is relatively straightforward. Typically, diagnostic arthroscopy is performed prior to the injection of MSCs in order to confirm and document the cartilage lesions present, following which, the preprepared solution of cultured MSCs is injected directly into the joint. However, a factor that has been postulated to be crucial in determining the efficacy of MSC injection for cartilage regeneration is that of the number of cultured MSCs introduced intra-articularly. A typical low dose of MSCs for injection is that of a magnitude of 10^6 , with medium doses being in the order of magnitude of 10^7 and high doses being in the order of magnitude of 10^8 (Jo et al. 2014). Several trials observed that increasing the number of cultured MSCs delivered via intra-articular injection directly correlated with improved radiological, histological, and arthroscopic outcomes (Kim et al. 2015a; Jo et al. 2014). However, no consensus was reached on the exact minimum number of MSCs required for optimal outcomes, with the limiting factor for routinely increasing the amount of cultured MSCs injected being increased cost and time required for culturing (Fig. 14.3).

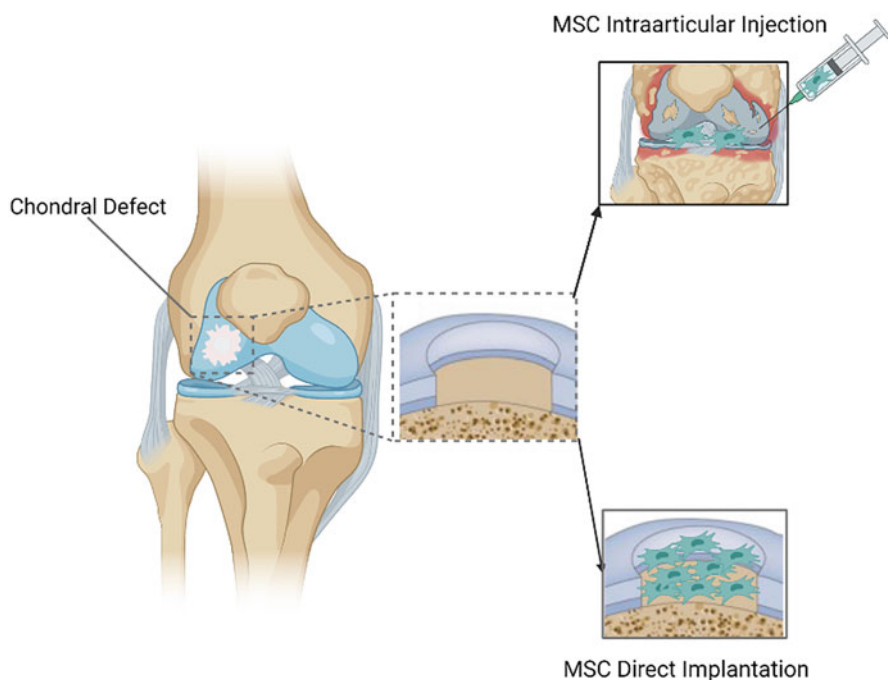


Fig. 14.3 MSC delivery techniques

14.5 Rehabilitation Following Cartilage Repair

Despite repair stability being a crucial hallmark of successful cartilage regeneration procedures, adequate postoperative protection of the cartilage lesion site is essential for good defect healing. However, due to the introduction of pro-hypertrophic MSCs to the joint, there is potential for development of undesirable side-effects such as arthrofibrosis after MSC introduction. As such, a delicate balance between axial offloading and joint movement is essential in order to maximize protection of the cartilage defect site and minimize the potential for arthrofibrosis development. Yet, to date no consensus exists regarding an established rehabilitation regimen for patients undergoing MSC-based cartilage regeneration procedures (Goldberg et al. 2017). Studies reporting the postoperative rehabilitation regimens of patients undergoing MSC-based cartilage regeneration procedures generally agree on a nil or partial weightbearing status for patients for at least four postoperative weeks, regardless of the MSC introduction technique (Wong et al. 2013; Koh et al. 2014; Kim et al. 2015b). Similarly, early joint mobilization exercises are initiated from the first postoperative day, most often by means of continuous passive movement machines or by exercises prescribed by physical therapists, with the aim to restore a functional range of motion in a controlled fashion. Upon returning to full

weightbearing status and the restoration of a good range of motion, the subsequent aim of rehabilitation is that of restoring muscular function in order to decrease the subsequent mechanical stresses exerted on the joint (Øiestad et al. 2022), with devices such as stationary bicycles, elliptical trainers, and closed chain exercises commonly employed as means of muscular resistance training. However, the effect of rehabilitation regimens on the efficacy of cartilage repair procedures is still not well established and ultimately should be tailored to suit the activity demands of individual patients.

14.6 Conclusion

Despite MSC-based therapies being utilized to treat cartilage diseases for almost two decades, preexisting cell-based treatment modalities such as ACI and MACI are well established and have been reported to produce stellar long-term outcomes (Ogura et al. 2017; Peterson et al. 2010). Additionally, the process of culturing and preparing MSCs for cartilage repair is expensive and time-consuming. Due to these factors, the integration of MSC-based therapies for cartilage regeneration into routine clinical practice has been slow, and as such, still remains a novel treatment modality for a majority of clinicians. However, with the increasing amount of high-quality evidence emerging supporting the efficacy of MSCs for the treatment of cartilage lesions, the novelty status of such therapies should be interpreted with the consideration that MSCs may yield the key to the next generation of personalized medicine for cartilage diseases.

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Extracellular Vesicles: A Potent Therapeutic Tool for Cartilage Regeneration 15

Nazmul Huda Syed, Maryam Azlan,
Muhammad Rajaei Ahmad Mohd Zain, Harishini Rajaratnam,
Nur Azira Mohd Noor, and Asma Abdullah Nurul

Abstract

Extracellular vesicles (EVs), which mostly include exosomes and microparticles are bilayer lipids that encapsulate nucleic acids and proteins. They participate in cell-to-cell communication, regulating a number of cellular processes, including cell development, proliferation, and migration. Studies conducted both in vitro and in vivo have shown that EVs have the ability to regenerate cartilage. Exosomes derived from mesenchymal stem cells (MSCs) have shown potential in preclinical studies for cartilage repair and cell-free therapies for cartilage degenerative diseases. Exosomes derived from mesenchymal stem cells (MSCs) or chondrocytes have been shown to stimulate chondrogenic differentiation and chondrocyte proliferation. MSC-derived exosomes serve as promising candidates in osteoarthritis cell-free therapy as well as in cartilage repair. Since mature chondrocytes in cartilage have limited regenerative capacity, effective therapeutics have been developed to repair and replace degenerating cartilage. However, obstacles such as the difficulties in standardizing targeted therapy have hindered EVs from being extensively employed as a treatment option. In this chapter, we provide insights into EV biology and their role in chondrogenesis, as well as describe the current therapeutic applications and future implications of EVs in cartilage regeneration.

N. H. Syed · M. Azlan · H. Rajaratnam · N. A. Mohd Noor · A. A. Nurul (✉)
School of Health Sciences, Health Campus, Universiti Sains Malaysia, Kubang Kerian, Kelantan,
Malaysia
e-mail: nurulasma@usm.my

M. R. Ahmad Mohd Zain
Department of Orthopaedics, School of Medical Sciences, Health Campus, Universiti Sains
Malaysia, Kubang Kerian, Kelantan, Malaysia

Keywords

Cartilage · Cell-free therapy · Exosome · Extracellular vesicles · Mesenchymal stem cell

15.1 Introduction

Cartilage is an alymphatic, avascular, and aneural connective tissue comprising chondrocytes and extracellular matrix (ECM). Cartilage degeneration can occur due to either trauma or degenerative disorders. Osteoarthritis (OA) is distinguished by the degeneration of cartilage over time, and is the most prevalent disease associated with cartilage, especially affecting the elderly (Martin and Buckwalter 2002). Chondrocytes secrete ECM and are the major component of cartilage. Their function and survival depend on a suitable environment, which is affected during a cartilage injury or degeneration resulting in chondrocyte death (Lee and Wang 2017). Moreover, there is currently no curative therapy for cartilage diseases despite their high prevalence.

Recently several treatment methods including microfracture therapy and cell-based therapy (autologous chondrocyte implantation) have been employed to treat cartilage degeneration. However, they pose several issues such as cell source availability, formation of fibrocartilage, and risk of graft rejection (Kaul et al. 2012). Therefore, the treatment of cartilage damage remains challenging. Stem cells are now frequently used in tissue healing as therapeutic cells. They are readily available, can proliferate and differentiate into specific cell types, and can replace the injured tissue. The importance of stem cells is credited to their secretion of paracrine factors (Zhang et al. 2020a). Extracellular vesicles (EVs), such as exosomes, are produced by cells including chondrocytes and fibroblasts, and these EVs represent the pathological alterations that occur during cartilage degeneration process. Moreover, due to their cargo containing proteins and RNAs, EVs play role in the cell-to-cell communications and cellular functions (Zhao and Xu 2018; Zheng et al. 2019). In addition, due to their excellent biocompatibility and low immune rejection, EVs have shown huge scope for diagnosis, regeneration, and repair of affected cartilage (Sun et al. 2019). EVs secreted by mesenchymal stem cells (MSCs) can stimulate regeneration of cartilage tissue in early OA patients (Zhang et al. 2018). Since many MSC functions are regulated by paracrine factors, exosomes have been suggested as a potential therapy for cartilage diseases. Therefore, the presence of EVs and their therapeutic properties enable clinical applications for cartilage repair and regeneration. Although numerous studies have shown that EVs are effective for cartilage repair, it is still unclear how EVs work and how they may be used therapeutically to promote cartilage regeneration. In this chapter, we provide information on cartilage, exosomes, and their role in chondrogenesis, as well as current strategies regarding the possible use of EVs as therapeutic targets for cartilage repair and regeneration.

15.2 Cartilage

Cartilage, like bone, is a specialized connective tissue that contributes to the overall body framework. Morphologically, cartilage is classified into three types: hyaline cartilage, fibrocartilage, and elastic cartilage. Hyaline cartilage is homogeneous and the most abundant type of cartilage including articular cartilage, which lines the articulating ends of bones in many synovial joints such as the knee, shoulder, hip, and other movable body joints, is the most studied type of cartilage. Fibrocartilage is fibrous, has a high tensile strength, and is prevalent in areas where there is significant mechanical stress. It forms the annuli, menisci, and plates of intervertebral discs, knees, and opposing bone joint surfaces, respectively. Compared to other types of cartilage, elastic cartilage is significantly more flexible. It is present in areas of the body that require stretchability, such as the epiglottis, external ear, and vocal cord attachment to the larynx (Williams et al. 1995; Bissell and Steele 2010; Benjamin and Evans 1990).

Chondrocytes constitute about 2% of mature human articular cartilage. The ECM comprises the majority of the tissue and relies on this small proportion of chondrocytes to produce and maintain articular cartilage throughout the lifetime. The loss of these cells will eventually lead to ECM fatigue and joint degeneration. During the early stages of articular cartilage development, the cartilage progenitor cells (CPCs) at the end of bone, go through a series of heavily controlled differentiation and maturation processes (Hunziker et al. 1987). Following the series of events such as angiogenesis, ECM mineralization, and apoptosis, CPCs undergo significant phenotypic changes that result in bone formation. Mature chondrocytes, on the other hand, have a stable phenotype of sizes (10–15 μm in diameter) (Pacifici et al. 2005). In terms of functionality, they are highly specialized cells that are accountable for ECM synthesis and remodeling, which regulate the mechanical and functional properties of articular cartilage (Buckwalter 1998). However, post-injury, mature chondrocytes exhibit morphological changes as well as differentiated gene expression patterns, affecting their capacity to restore or maintain the ECM. Mesenchymal stem cells (MSCs) are another cellular component of cartilage. MSCs have been proposed for the development of engineered cartilage tissue as a replacement for mature chondrocytes, which are incapable of regenerating or repairing damaged cartilage. They are present in both healthy and osteoarthritic articular cartilage of the human body. Tissue-specific MSCs in diverse adult tissues can differentiate, proliferate, and regenerate affected tissue in the event of injury (Alsalameh et al. 2004; Slack 2008).

15.3 Cartilage Injuries

Articular cartilage injury is a common diarthrodial joint disorder that can cause significant musculoskeletal morbidity and secondary osteoarthritis if not treated properly. It can affect people of all ages, resulting in a wide range of clinical manifestations and disabling conditions that represent a significant and growing

health concern with significant implications for the affected individual (Hunter and Bierma-Zeinstra 2019). The articular cartilage structure allows smooth gliding in the joint as well as contributing to effective shock absorption. The unique and intricate framework of articular cartilage makes it difficult to treat and restore defects. Lacking blood vessels and cells in the tissue causes the limitation in response to repair defects of any size (Fritz et al. 2008).

Depending on the site of the injury, cartilage injuries can be classified as either superficial or deep lacerations, which extend either superficially to the tidemark or through the tidemark to the subchondral bone. The mechanism for cartilage repair depends on where the injury occurred and involves both the cartilage and the surrounding tissue. The cartilage attempts to repair itself in superficial or partial-thickness lacerations with the least amount of support from the surrounding tissue. Chondrocytes proliferate, and a tiny proportion of synovial fibroblasts migrate to the injury, but there is no actual healing owing to the lack of hemorrhage and inflammatory response. In full-thickness injuries, the subchondral bone reacts by exhibiting a substantial hematoma and stem cell migration in significant numbers. However, these undifferentiated bone marrow MSCs can only generate type 1 collagen and not hyaline cartilage-like tissue. Due to this, fibrocartilaginous reparative tissue that is mechanically unsatisfactory and insufficient for long-lasting structural bonding with the surrounding, healthy hyaline cartilage is formed. As a result, fibrocartilaginous reparative tissue is generated, which is mechanically unsatisfactory and unsuitable for long-term structural bonding with the nearby, healthy hyaline cartilage. If left untreated, cartilage defects frequently progress to larger, higher-grade lesions over time, ultimately leading to osteoarthritis. OA is the most prevalent type of degenerative joint disease, characterized by cartilage degeneration and osseous overgrowth. In contrast to rheumatoid arthritis, OA is commonly viewed as a degenerative disease. The degenerative theory, however, is no longer acceptable given mounting evidence that inflammation also contributes significantly to the development and progression of OA (Nurul et al. 2021; Mohd Noor et al. 2021).

15.4 Current Treatments and Challenges in Management of Cartilage Injuries

Once trauma or disease initiates an intra-articular degenerative process, adult articular cartilage has a limited capacity to repair itself, especially for larger defects (Mont et al. 2000). This is due to the chondrocytes' inability to migrate to the injury site, the lack of a fibrin clot scaffold, and the avascular nature of the tissue that led to the limited ability of the cartilage to self-heal.

Several different techniques for treatment have already been discussed, depending on the location and extent of the lesion, as well as the patients' level of activity. Factors to consider include age, the duration of the ailment, the location and depth of the injuries, and other factors. They cover a wide range of treatment options, from nonsurgical therapies to joint preservation and replacement surgery. Although there are several techniques to repair cartilage defects, the majority of them are

symptomatic in nature and do not mimic normal joint function to promote long-term healing. These procedures have different degrees of effectiveness, but they are not without drawbacks, especially as the repaired area's characteristics are often inferior to those of the surrounding normal cartilage and often do not integrate with them (Pape et al. 2010).

Multiple surgical procedures for treating localized chondral lesions have been described and can be categorized as repair, reconstruction, and regenerative approaches (Forriol 2009). The repair methods (perforation and microfracture) promote the growth of fibrocartilaginous tissue, making it easier for blood vessels and osteoprogenitor cells to access the site and initiate chondrogenesis. Reconstruction techniques aim to repair the injury using autologous articular cartilage transplantation or allografts (OATS or osteochondral autograft transfer, mosaicplasty, allografts) through arthroscopy or mini-arthroscopy. Meanwhile, regenerative techniques using bioengineering methods such as autologous chondrocyte implant (ACI), MSCs, or chondrocytes in different scaffolds (MACI) are being used to generate hyaline cartilage tissue (Vaquero and Forriol 2012).

15.5 EV Biology

15.5.1 Biogenesis

Extracellular vesicles (EV) are lipid nanomembrane particles comprising microparticles, exosomes, and apoptotic bodies (Yáñez-Mó et al. 2015; Zaborowski et al. 2015). Each EV subtype has its own distinct biological pathway for secretion and characteristics (Fig. 15.1). Exosomes are produced when cargo fuses with the plasma membrane to form early endosomes. The cytoplasmic content is then secreted into the surrounding lumina to form intraluminal vesicles (ILVs) (McAndrews and Kalluri 2019). The ILVs budding into the lumen to form late endosomes, also known as MVBs through the activation of protein complexes such as the endosomal sorting complex required for transport (ESCRT) dependent pathway and independent pathway. The ESCRT has four complexes (ESCRT 0, I, II, and III) that work with unique functionality in different pathways.

In the ESCRT-dependent pathway, initial inactive ESCRT-0 is converted to a heterotetramer via phosphatidylinositol 3-phosphate (PI-3-P) intended for the clustering of ubiquitinated cargo on the outside of the endosomal membrane. The active ESCRT-0 recruits the ESCRT-I via the interaction of hepatocyte growth factor-regulated tyrosine kinase substrate prosaposin (HRS PSAP) domains and the ESCRT-I subunit tumor susceptibility gene (TSG) 101 (Hessvik and Llorente 2018). At the same time, the ESCRT-I binds with the ESCRT-II of vacuolar protein sorting-associated protein (VPS) 36 and 28 domains to be activated for delivery of the ubiquitin-tagged proteins to the ESCRT-III (Babst et al. 2002). The three protein subunits [Sucrose nonfermenting (SNF) 7, VPS 20, and VPS 4] of ESCRT-III are responsible for the scission of intraluminal vesicles (Hurley and Hanson 2010; Wollert and Hurley 2010). The VPS20 binds directly to the ESCRT-II and activates

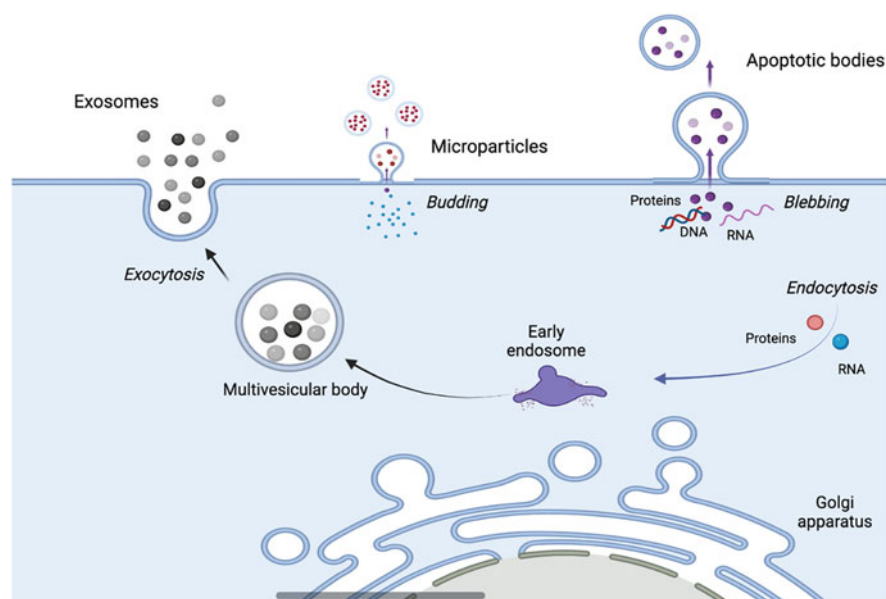


Fig. 15.1 Biogenesis of EVs including exosomes, apoptotic bodies, and microparticles. (Created using [BioRender.com](https://www.biorender.com))

the SNF7 for the cleaving of vesicle neck constriction. The VPS4 mediates the dissociation of the ESCRT complexes and releases the vesicle from MVBs to the cytosol. The MVBs are fused with the lysosome for degradation or coupled with the GTPases Ras-associated proteins (RAB) 27A, 27B, 35, 2B, 11, 4, 7, 5A, 9A, and 7 for regulating exosome secretion and release (Tschuschke et al. 2020). Still, the details of their actions remain unknown. The soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) enable the MVBs exocytosis by facilitating their fusion with the plasma membrane (Ståhl et al. 2019).

Microparticles (MP) originate from tightly packed heterogeneous microdomains that directly outward blebbing the plasma membrane into extracellular space. The generation of MP involves cytoskeleton remodeling and aminophospholipid (APL) externalization. Initially, the ATP-dependent transmembrane enzymes flippase (moving in from extracellular space) and floppase (moving out to extracellular space) maintain plasma membrane symmetry (Qiao et al. 2021). Both works are complementary and catalyze the movement of phospholipids within the plasma membrane. As the cell is damaged, it leads to an influx of intracellular calcium (Savina et al. 2003). The calcium ions will inhibit the flippase activity and activate the scramblase activity that leads to the aminophospholipid rapidly redistributing across the bilayer and losing its symmetry (Qiao et al. 2021). The intracellular signaling event resulted from the activation of calpain, rho kinase, and transglutaminase for cytoskeleton destruction and binding with the plasma membrane (Said et al. 2018). This rebuilding of cytoskeleton will result in the outward

blebbing of the plasma membrane. As a result, the membrane conformation changes and leads to the shedding of MP (Burnier et al. 2009). Lastly, apoptotic bodies arise either from the separation of the membrane blebs or from the cell during the process of apoptosis. Typically, the process begins with the chromatin of the cell contents clumping, and then the cell membrane is ruptured to produce a variety of vesicles. However, the role and route of these particles remains unclear (Margolis and Sadovsky 2019).

15.5.2 Characteristics

EVs exist in various sizes, shapes, and contents/cargoes. The majority of EVs transport biomolecules such as genomic DNA, mitochondrial DNA (mtDNA), and plasmid DNA as transferring agents locally and systematically (Lázaro-Ibáñez et al. 2014; Grüll et al. 2018; Erdmann et al. 2017). DNA-EVs range in size from 100 bp to 20 kbp, reflecting the mutational status of the tumor parental cells (Amintas et al. 2021). As a result, the extracted DNA from all EV groups represents a promising biomarker for tumor disease state and complexity. Exosomes, on the other hand, are the smallest nanomolecules, with a typical diameter of 30–150 nm and a cup/round shape (Qiao et al. 2021; Jung and Mun 2018). Many studies have found biomolecules such as messenger RNA (mRNA), long noncoding RNA (lncRNA), and microRNA (miRNA) are also loaded inside the exosome. All these act as mediators to regulate the physiological properties of the cell, enabling a therapeutic potential similar to stem cells in cartilage tissue engineering (Zhou et al. 2020). However, few reports propose DNA-free exosome composition, which contradicts current information (Jeppesen et al. 2019). Recently, studies have implanted and explained the vigorous preparation and isolation EV method as attributed to the yield and quality of exosomes (Shen et al. 2020). Some studies discovered that the ESCRT protein accessory (Alix, TSG101, Heat shock cognate (HSC) 70, and heat shock protein (HSP) 90B) and tetraspanin protein (CD63, CD9, CD81) are well enriched on the surface of exosomes and are referred to as protein markers (Tauro et al. 2012; Morita et al. 2007; Théry et al. 2001). Exosomes are also found to contain the lipids (sphingomyelin, ceramide) that help exosomes maintain their bilayer structure (Zhou et al. 2020).

MPs are a diverse group of vesicles with diameters ranging from 100 to 1000 nm and irregular/fragmented shapes (Bian et al. 2019). Similarly, it carries the same bioactive properties as exosomes that support cartilage repair and regeneration. ADP ribosylation factor 6 (ARF6) and vesicle-associated membrane protein 3 (VCAMP3) have recently been proposed as markers that have emerged on the surface structure model of microvesicles (Bian et al. 2019). The bioactive lipid cargoes (cholesterol and sphingolipid) within the microparticle modulate the bilayer structure of membrane vesicles in the same way that exosomes do. Lastly, apoptotic bodies are reported as the largest EVs, measuring 1000 to 5000 nm in diameter and having a round shape. Interestingly, in contrast to exosomes and MPs, apoptotic bodies contain organelles, chromatin, and small amounts of glycosylated protein.

15.6 Involvement of EVs in the Pathophysiology of Cartilage Diseases

Among EVs, exosomes are the most studied and are regarded as more important in tissue engineering and regenerative medicine due to their size and composition. Exosomes are secreted into the body fluids by almost all cells, including blood cells, immune cells, and mature somatic cells. Each exosome's cargo is reflective of its origin cells, which regulate exosome-cell interactions (Tavasolian et al. 2021). Synovial fluid (SF) contains exosomes, and their composition and functions are altered during a cartilage injury or degeneration. Differential expression of proteins and miRNAs has been extensively reported in the SF of patients with OA (Zhao and Xu 2018; Kolhe et al. 2017). Exosomes from fibroblast-like synoviocytes have been shown to increase inflammatory cytokine levels and activate CD4+ cells in patients with OA and rheumatoid arthritis (Withrow et al. 2016). Furthermore, they have been associated with cellular functions such as mediating bone and cartilage degradation, promoting osteoclast function, stimulating pro-inflammatory cytokines, and increasing the influx of immune cells in patients with cartilage diseases, which further degrade the cartilage. Numerous studies have been conducted in recent years to look into the possible use of exosomes as diagnostic markers and in gene delivery for therapeutic reasons. Additionally, it is used to minimize immunological rejection during the transplantation of cells and organs by suppressing immune responses. Because exosomes play critical roles in the control of inflammation and immunological responses, they have been extensively utilized in the treatment of OA and other autoimmune and inflammatory conditions (Song et al. 2021).

15.7 Chondrogenic Role of EVs

Multiple sources of MSCs are extensively utilized to differentiate into mature chondrocytes in cartilage tissue engineering (Hosseini et al. 2019), while few have utilized growth factors and miRNAs to induce chondrogenic differentiation (Mahboudi et al. 2018). MSC-derived exosomes were shown to promote chondrogenic differentiation and tendon stem/progenitor cell (TSPC) proliferation into mature chondrocytes in vitro (Yu et al. 2020). Exosomes and microparticles derived from MSCs have been shown to protect osteoarthritis-derived murine chondrocytes in vitro and in vivo. Exosomes extracted from murine bone marrow-derived MSCs (BM-MSCs) were found to enhance the chondrocyte markers' expression such as type II collagen and aggrecan. In contrast, exosomes suppressed the expression of immune and inflammatory components such as matrix metalloproteinases (MMPs), which are responsible for cartilage degradation. Chondrocytes activated by exosomes were unable to stimulate CD4+, CD8+, or B lymphocytes in vitro (Cosenza et al. 2017). In concordance, another study reported that MSC-derived exosomes suppressed the chondrocyte apoptosis and promoted their proliferation. Furthermore, exosomal lncRNA-KLF3-AS1 was reported to inhibit miR-206, which promoted the expression of G-protein-coupled receptor

kinase interacting protein-1 (GIT1) in chondrocytes (Liu et al. 2018). Subsequent studies have reported that GIT1 regulated chondrocyte proliferation and prevented chondrocyte apoptosis (Zhang et al. 2015). MSC- and chondrocyte-derived exosomes contain substances that stimulate chondrocyte proliferation and migration, as well as guide stem cell/progenitor chondrogenic differentiation.

Jing et al. demonstrated that miR-381-rich small EVs (sEVs) promoted stem cell chondrogenesis (Sun et al. 2019). This study found that miR-381-3p inhibited TAOK1, which in turn inhibited the Hippo signaling pathway that induces apoptosis and inhibits cell proliferation. Exosomes derived from MSCs with elevated levels of miR-92a-3p enhanced the migration and proliferation of chondrocytes, according to Mao et al. (Mao et al. 2018). Exosomal miR-92a-3p was found to inhibit WNT5A, also known as Wnt family member 5A, an essential component in the pathogenesis of OA. Sun et al. analyzed the miRNA profile of exosomal miRNAs during stem cell chondrogenesis. A total of 34 miRNAs, including miR-320c, were reported to be upregulated. After transfecting hBMSCs with miR-320c, they discovered that hBMSC-320c-Exos are more effective in promoting chondrocyte proliferation and stem cell differentiation. MiR-320c targets inflammatory mediators like MMP and Runt-related transcription factor 2 (RUNX2), which suppress their expression during cartilage breakdown (Sun et al. 2019; Meng et al. 2016).

Furthermore, miR-8485 derived from chondrocytes has also been shown to promote the differentiation of BM-MSC into chondrocytes. By targeting glycogen synthase kinase 3 beta (GSK3B), miR-8585 activates Wnt/ β -catenin pathway. Thus, promoting chondrocyte differentiation from stem cells (Li et al. 2020). Another recent study used circulating exosomes from blood to transfect rabbit BM-MSCs with miR-140 to demonstrate that exosomes, together with miR-140, significantly promoted differentiation of BM-MSCs to chondrocytes (Won Lee et al. 2020).

15.8 Role of EVs in Cartilage Repair

Cartilage degeneration often occurs due to chronic inflammatory diseases or degenerative joint diseases and trauma. Cartilage damage affects the number of chondrocytes by inducing apoptosis, thus allowing ECM degradation faster than it is produced. Therefore, deep insights into the pathogenesis of cartilage damage may facilitate further research on developing therapeutic options.

Traumatic cartilage damage induces synoviocyte and chondrocyte stress, which leads to the ECM degradation of cartilage, inflammation, and apoptosis. Inflammation due to cartilage injury causes an increase in pain and the progression of cartilage degradation (Schulze-Tanzil 2009). An imbalance in the anabolic and catabolic pathways of the ECM contributes to the inflammation. Inflammatory cytokines such as IL-1 β , TNF- α , IL-6, IL-15, IL-17, and IL-18 are frequently responsible for cartilage inflammation (Wojdasiewicz et al. 2014). The inflammatory regulators are known to be inhibited using EV treatment. Zhang et al., for example, demonstrated that exosomes derived from MSCs reduced IL-1 β expression, which is a key pro-inflammatory regulator (Zhang et al. 2019). A more recent study demonstrated

that exosomes derived from MSCs hindered the advancement of intervertebral disc degeneration via inhibiting the activation of inflammatory regulators and the NLRP3 inflammasome (Xia et al. 2019). By inhibiting the NLRP3 pathway, pyroptosis, which is a programmed cell death, can be prevented. MSC-derived miR-410 is reported to suppress the NLRP3 pathway, thus regulating pyroptosis (Zhang et al. 2020b). Recently, Zhang et al. reported that BMSC-derived exosomes decreased the inflammation via mediating macrophage polarization, inhibiting M1 (pro-inflammatory) macrophages, and increasing M2 (anti-inflammatory) macrophage production (Zhang et al. 2020c).

Components of the ECM, such as collagen type II and proteoglycans, regulate chondrocyte functions. These ECMs can be synthesized via therapy. In an animal model of OA, He et al. showed that exosomes from BMSCs enhanced the production of collagen type II while decreasing the expression of MMP13 (He et al. 2020). BMSC-derived exosomes were further shown to contribute to the production of ECM in vitro. It would therefore appear that EVs play specific roles in the process of recovering ECM. Furthermore, miRNAs (miR-8485), one of the key constituents of EVs, is reported to promote BMSC differentiation into chondrocytes via activating Wnt/ β -catenin pathways (Li et al. 2020).

In addition, the loss in chondrocyte count due to autophagy and apoptosis could be addressed with EV therapy. For example, miR-21 in exosomes derived from MSCs is shown to inhibit apoptosis in the nucleus pulposus cells (Cheng et al. 2018). Similar research has suggested that EVs can be used to prevent apoptosis and promote cell proliferation in intervertebral disc degeneration to sustain the number of chondrocytes (Xiang et al. 2020). The findings suggest that the role of EVs is to maintain the homeostasis of cartilage through preserving the chondrocyte number, as well as balancing the ECM metabolism. The role of EVs in cartilage tissue repair is illustrated in Fig. 15.2.

15.9 EV-Based Cell-Free Therapy for Cartilage Repair

Cellular therapy using MSCs has been widely employed in cartilage repair and tissue engineering, as MSCs are useful therapeutic tools due to their differentiation capability. Nevertheless, several contraindications to MSC-based therapy include immunogenicity (Cho et al. 2008), and tumorigenicity-related complications particularly due to direct MSC transplantation (Beckermann et al. 2008). Therefore, MSC-derived exosomes have become a promising therapy for cartilage regeneration in place of MSC. Numerous types of cells, including blood cells, immune cells, tumor cells, as well as MSC, generate exosomes. Exosomes are an important biochemical messenger that facilitates cellular communication by transmitting the data via bioactive components found in the membrane and cytoplasm, as well as nucleic acids, lipids, and proteins (Yin et al. 2019). Beside expressing lactadherin, tetraspanins (CD81, CD63, and CD9), integrins, and heat-shock proteins (HSP60, HSP70, and HSP90) which allow them to incorporate into recipient cells (Heo and

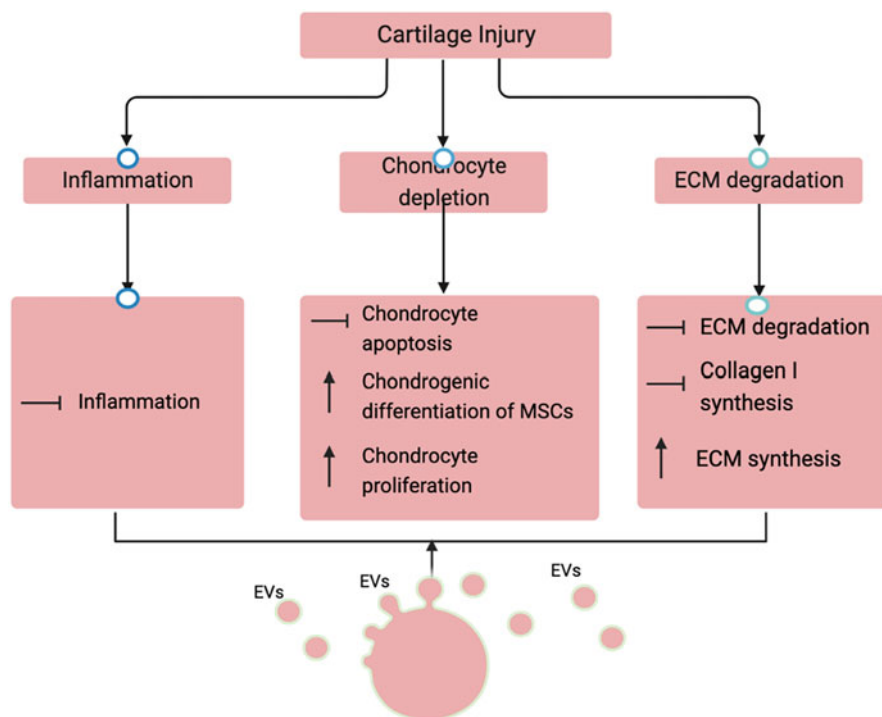


Fig. 15.2 Illustration of the EVs' role in cartilage repair. (Created using [BioRender.com](https://www.biorender.com))

Kim 2018), these nanovesicles also express specific markers that reflect their cellular origin (Schey et al. 2015).

Exosomes derived from MSCs are thought to regulate the activation of the AKT, ERK 1/2, and AMPK signaling pathways primarily through the activity of CD73, which has been implicated in the regulation of the therapeutic effects of exosomes (Zhang et al. 2018). During inflammation, ATP and ADP released by injured chondrocytes were hydrolyzed into AMP, in which CD73-expressed exosomes may further hydrolyze AMP into adenosine, a crucial kinase activator. This hydrolysis activity subsequently resulted in activation of adenosine receptor, which leads to the phosphorylation of the AKT, ERK 1/2, and AMPK signaling pathways in chondrocytes (Colgan et al. 2006). This process further promotes necessary cellular responses in cartilage repair, including chondrocyte differentiation, proliferation, and migration (Chew et al. 2019). Furthermore, it has been previously reported that MSC-derived exosomes exhibit a chondroprotective effect and prevent ECM degradation through their immunomodulatory function. Immunomodulatory contents of exosomes such as IFN- γ , HGF, and TGF- β allow them to modulate immune activity through paracrine effects (Chen et al. 2014), thus providing a balance between pro- and anti-inflammatory effects to maintain cartilage homeostasis.

Exosomes derived from MSC have shown a promising chondrogenic effect by promoting chondrocyte differentiation and proliferation, highlighting their role in cartilage regeneration and tissue engineering. Chondrocyte differentiation is one of the critical processes in cartilage repair, and exosome have always been suggested to enhance chondrocyte differentiation in vitro through their miRNA activity (Song et al. 2021). For instance, human BMSC-derived exosomes expressing miR-320c promoted chondrogenic differentiation and chondrocyte proliferation by hindering MMP-13 and Runt-related transcription factor 2 (RUNX2), both of which are responsible for inflammatory activity during degeneration of cartilage in OA (Sun et al. 2019). Others have reported that MSC-derived exosomes expressing miR-92a-3p stimulated chondrogenic differentiation in human MSC and primary human chondrocyte (PHC) models (Mao et al. 2018). Further inhibitory assays using anti-MSC-derived exosomes expressing miR-92a-3p resulted in the expression of WNT5A, thus decreasing chondrogenic differentiation and the synthesis of cartilage matrix. Additionally, a recent study has shown that BMSC-derived exosomes enhanced the tenogenic differentiation, proliferation, and migration of tendon stem/progenitor cells (TSPC) in the patellar tendon defect of rat model (Yu et al. 2020). Inhibition of exosomes using exosome inhibitor GW4869 resulted in impedance of exosomes secretion, subsequently impacted the migration and proliferation of TSPC by BMSC.

Chondrocyte proliferation is another crucial process in cartilage repair to achieve therapeutic benefit, as an increase in cell mass as well as an increase in ECM synthesis may mitigate OA complications (Toh et al. 2016). Previous findings have demonstrated that MSC-derived exosomes could potentially promote chondrocyte proliferation and prevent apoptosis by attenuating IL-1 β -induced chondrocyte injury through exosomal KLF-AS1 activity (Liu et al. 2018). By inhibiting miR-206, these exosomes were demonstrated to control the expression of G-protein-coupled receptor kinase interacting protein-1 (GIT1) in chondrocytes, thereby promoting chondrocyte proliferation and preventing apoptosis. Other studies have also shown that MSC-derived exosomes may promote chondrocyte proliferation. Intra-articular injection of MSC-derived exosomes resulted in a significant elevation of the proliferation marker proliferative cell nuclear antigen (PCNA) in the synovium and reparative tissue in the rat model compared to the control group (Zhang et al. 2016). Injection of MSC-derived exosomes into reparative tissue also resulted in the reduction of apoptotic cells and the polarization of macrophages with the M2 phenotype without triggering any destructive immune responses. Meanwhile, injection of hBMSC-derived exosomes treated with miR-26a-5p in rats with OA enhanced synovial cell proliferation and infiltration of synovitis cells and reduced the expression of MMP-3 and MMP-13 in synovial tissue (Jin et al. 2020).

In addition to chondrocyte differentiation and proliferation, MSC-derived exosomes may exert other therapeutic effects that are beneficial in cartilage repair, including attenuation of inflammation and improvement of cellular bioenergetics. It has been shown that BMSC-derived exosomes can reduce the production of pro-inflammatory cytokines and suppress the activation of macrophages, the production of nitric oxide, and the synthesis of MMP-13, all of which contribute to

inflammation in OA (Ren et al. 2019). MSC-derived exosomes are also involved in mTOR pathway inhibition through the activity of miR-100-5p, thus aiding in the prevention of cartilage inflammation (Zhang et al. 2013). Meanwhile, exosomes also exhibit restorative effects by enhancing mitochondrial bioenergetics. Previous reports have suggested that exosomes contain several glycolytic enzymes such as adenylate kinase, phosphoglucokinase, and pyruvate kinase, which are important in inducing the synthesis of ATP in chondrocytes (Arslan et al. 2013). Diminished mitochondrial electron transport chain (ETC) proteins and mitochondrial dysfunction which lead to low level of chondrocyte bioenergetics, have always been observed in OA conditions. A lowered expression of ETC proteins resulted in low ATP production, reduced ECM synthesis, and the generation of oxidative stress (Zheng et al. 2019), which exacerbates inflammation in OA. Therefore, the use of exosomes in cell-free therapy may be useful in attenuating inflammation and regenerating cartilage. Figure 15.3 shows an outline of the therapeutic approaches using EVs for cartilage regeneration.

15.10 Future Perspectives and Conclusions

In conclusion, growing evidence proves the exosomal role in cartilage regeneration and chondrogenesis. Exosomes derived from both chondrocytes and MSCs promote stem cell progenitors' differentiation into mature chondrocytes *in vitro*. In addition, these exosomes stimulate the proliferation, differentiation, and migration of cartilage progenitor cells into chondrocytes. MSC-derived exosomes enhance bioenergetic levels and chondrocyte cell proliferation in the affected cartilage. Furthermore, they increase the levels of immunoregulatory and anti-inflammatory cell mediators while decreasing pro-inflammatory cytokine expression. As a result, the application of MSC-derived exosomes as a cell-free therapy option presents a significant opportunity for treating OA and other degenerative diseases. The mechanisms by which EVs contribute to cartilage regeneration, however, are still unknown and need to be better understood through further research. Nonetheless, it should be noted that efficient and optimal isolation as well as the purification of exosomes are still challenging. Each sample source has varying concentrations of exosomes. Therefore, it is suggested to optimize the isolation protocols of exosomes prior to their application. Furthermore, adequate cartilage regeneration is difficult when using EVs from varying sample sources.

For efficient and effective EV therapy, it is necessary to identify parental cells and develop therapeutic strategies. Through maintaining the homeostasis of cartilage, therapies based on EVs have the potential to replace damaged cartilage. EVs can be designed and encapsulated with various biomaterials to control their release and enhance their therapeutic effects. Appropriate approaches will increase the accessibility and efficacy of EV therapeutic interventions for cartilage regeneration. For clinical applications, the EV-based strategies must be standardized to ensure their biocompatibility in the future.

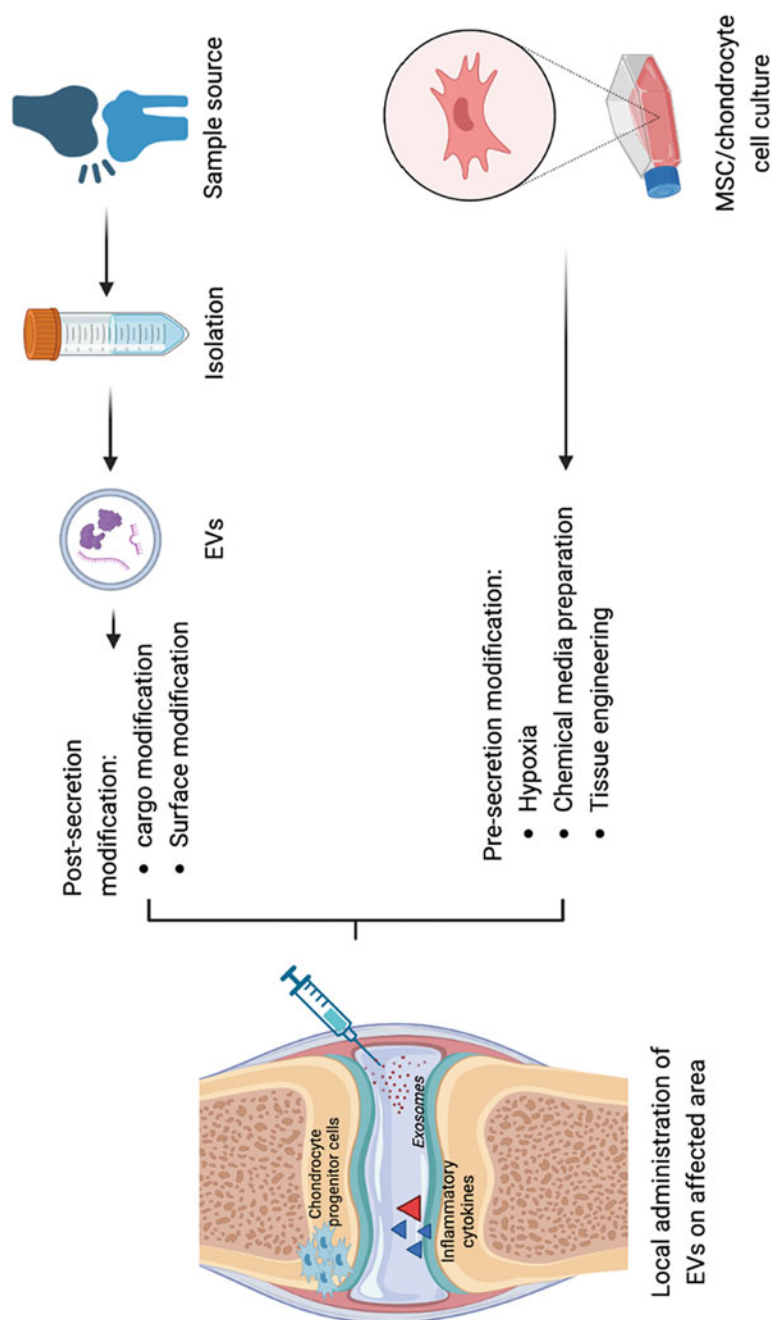


Fig. 15.3 Treatment strategies for cartilage repair using EVs. EVs are administered into the affected area for cartilage repair and replacement after being treated post- and pre-secretion for any modifications from body fluid, tissue, chondrocytes, or MSCs. (Created using [BioRender.com](https://www.biorender.com))

Acknowledgments This study was supported by the Fundamental Research Grant Scheme (Project Code: FRGS/1/2021/SKK0/USM/03/7) from the Ministry of Higher Education Malaysia. The funding body had no role in study design or in the collection and interpretation of data.

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Osteochondral Unit Approach for Articular Cartilage Regeneration

16

Yanli Cai, Soo Wah Gan, Wen Feng Lu, and Ching-Chiuan Yen

Abstract

It is of great challenge to repair and regenerate osteochondral defects (OCDs) as the poor self-healing ability and spatial complexity of osteochondral units. Current non-surgical and surgical treatments for OCDs are unsatisfactory and encounter many limitations, difficulties and risks. Further development of more effective, targeted therapeutic approaches is necessary to repair and regenerate the osteochondral unit. The primary tissue engineering (TE) strategy is to mimic the native osteochondral structure and composition to induce zone-specific tissue regeneration. The fundamental requirements of osteochondral scaffolds are good biocompatibility, bioactivity and surface topography, appropriate biodegradability, adequate mechanical strength and suitable architecture and porosity. By careful selection of biomaterials, traditional TE approaches were adopted for osteochondral regeneration, including cell-free and cell-seeding, cell-based and scaffold-free approaches. With the emerging of three-dimensional (3D) printing technology, the hierarchical architecture of the osteochondral unit was precisely

Y. Cai · S. W. Gan

NUS Centre for Additive Manufacturing (AM.NUS), National University of Singapore, Singapore, Singapore

W. F. Lu

NUS Centre for Additive Manufacturing (AM.NUS), National University of Singapore, Singapore, Singapore

Department of Mechanical Engineering, National University of Singapore, Singapore, Singapore

C.-C. Yen (✉)

NUS Centre for Additive Manufacturing (AM.NUS), National University of Singapore, Singapore, Singapore

Division of Industrial Design, National University of Singapore, Singapore, Singapore

e-mail: didyc@nus.edu.sg

mimicked with biphasic, multiphasic and even gradient scaffolds. It is expected that further advancing TE approaches will achieve clinical translation of osteochondral scaffolds without life-long treatment or revision surgeries.

Keywords

Osteochondral unit · Articular cartilage · Subchondral bone · Tissue engineering · 3D printing

16.1 Introduction

Articular cartilage connecting with subchondral bone reduces friction between adjacent bones and provides load transfer across joints for general joints and skeletal movement. The relationship between the articular cartilage and subchondral bone is significant in maintaining healthy joints. They are usually considered as a whole unit named the osteochondral unit, where the hyaline articular cartilage anchors to the subchondral bone through a calcified cartilage layer (Fig. 16.1). The osteochondral unit withstands a combination of compressive, tensile and shear stresses. A tidemark is an interface between the hyaline and the calcified cartilage, resisting the articular shearing. With the characteristics of both cartilage and bone, the calcified cartilage shows very good structural integration and transmits mechanical loadings between the hyaline cartilage and the subchondral bone during joint movement. The subchondral bone plays an essential part in shock-absorbing, load transferring and nutrition supplying for overlaying cartilage (Mosher 2009; Lepage et al. 2019; Wei and Dai 2021).

Permanent harm and dysregulation of the osteochondral unit can adversely affect joint function. Osteochondral defects (OCDs) of the knee are often traumatic, with various degenerative lesions. When involving the osteochondral unit, lesions are challenging to handle as the poor self-healing ability of the articular cartilage and the subchondral bone. There is a high chance of osteoarthritis (OA) with untreated lesions (Lepage et al. 2019; Kon et al. 2020). However, there are no medicines to treat the lesions. Surgical restoration is still the only option for focal OCDs or severe joint diseases. Generally, surgery is highly complex, depending on the specific structure-mechanical property and crosstalk within the osteochondral unit and surrounding tissues (Lepage et al. 2019). Articular lesions could be restricted to the superficial cartilage or extended to the underlying subchondral bone. Any care regarding the articular surface lesion tends to fail without intact support from the subchondral bone. Along with a better understanding of the relationships between the articular cartilage and the subchondral bone, more attentions are to the restoration of physiological properties of the whole osteochondral unit rather than the articular cartilage only (Gomoll et al. 2010).

This paper reviewed current approaches for repairing and regenerating the osteochondral unit, including clinical treatment methods, requirements and biomaterials of osteochondral scaffolds, traditional tissue engineering

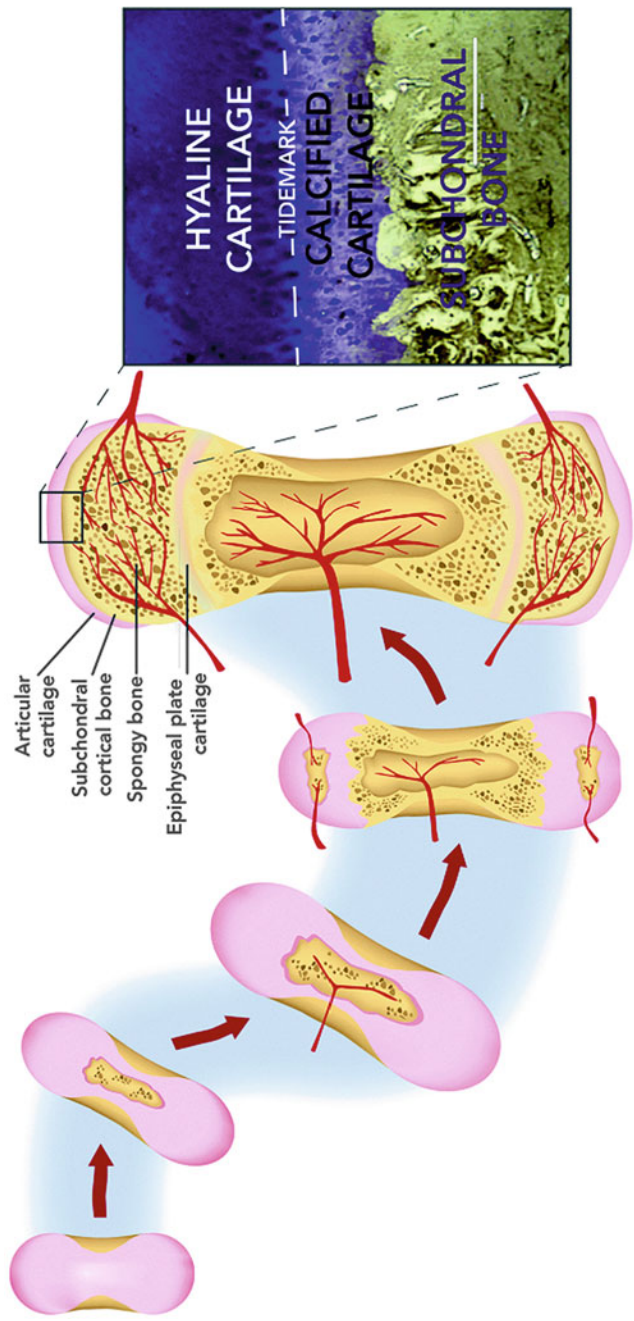


Fig. 16.1 Illustration of endochondral ossification and osteochondral unit. Inset: Toluidine blue (TB) and von Kossa staining of the osteochondral unit. Reproduced under the terms of the CC-BY license (Lepage et al. 2019). Copyright 2019, the Authors. Published by Mary Ann Liebert, Inc.

(TE) approaches and 3D printing strategic technology in the osteochondral unit. Finally, the challenges and prospects of osteochondral unit approaches were presented.

16.2 Clinical Treatment Methods

Due to the inherent poor regeneration capability, it is a severe problem for clinicians to handle OCDs. Before the surgical intervention, non-surgical treatments could temporarily relieve symptoms and slow joint disease progression. In case of unsuccessful conservative treatment, surgical treatments are adopted, which can be characterised as palliative, reparative and regenerative/restorative treatments (Wei and Dai 2021; Zhou et al. 2020; Tetteh et al. 2012).

The typical palliative treatments are arthroscopic debridement and lavage. It aims to reduce the pain, inflammation and mechanical irritation within the joint without the replacement of the injured tissue. It is the least invasive surgery that washes and removes the cartilage or bone debris and inflamed synovium (Wei and Dai 2021; Tetteh et al. 2012). There are controversial opinions regarding the effect of arthroscopic debridement. No difference was found between arthroscopic and sham surgery or optimised medical and physical therapy (Johnson 2002; Thorlund et al. 2015). A systematic review and meta-analysis focussing on the use of arthroscopic surgery for degenerative knee claimed poor benefits and harm after 2 years of partial meniscectomy, debridement or both for middle-aged or old patients with knee pain and degenerative knee disease (Thorlund et al. 2015). However, arthroscopic debridement and lavage were shown to relieve the pain significantly in subjects with OA when the treatment was done during the acute stage of degeneration (Jackson and Dieterichs 2003). In patients with early degenerative knees, arthroscopic debridement showed good symptomatic relief with sustained benefits (Law et al. 2019).

Microfracture is the most widely used reparative technique for treating osteochondral lesions. It is a marrow-stimulating procedure carried out by subchondral bone perforation to introduce autologous mesenchymal stem cells (MSCs) to a cartilage defect. Blood and MSCs from the underlying marrow cavity effectively stimulate the growth of new fibrocartilage that covers and remodels the damaged area. The fibrocartilage tissue is unlike the native hyaline cartilage, yet it still protects the bone surface during movement. The symptoms like pain and swelling are therefore alleviated. Microfracture surgery is minimally invasive, time and cost-effective and has a significantly shorter recovery time than an arthroplasty. It is regarded as the initial course of treatment for osteochondral lesions and has demonstrated effective short-term functional improvement. However, the fibrocartilage infill and the altered subchondral bone might negatively affect long-term osteochondral regeneration and lead to chronic defects and cartilage defects (Gomoll et al. 2010; Song and Park 2019; Erggelet and Vavken 2016).

Osteochondral transplantation has been applied to reconstruct severe OCDs for more than 20 years. It includes autograft and allograft transplantation with different

sources of donors. The donor of the autograft is the patient himself/herself, while the allograft is from other persons. Autograft transplantation has advantages such as a single-stage procedure, low cost, avoidance of disease transmission, disadvantages of availability of transferable non-essential cartilage and morbidity of donor site (Marcacci et al. 2013; Inderhaug and Solheim 2019). When the autografting procedure was appropriately performed, satisfactory outcomes could be achieved in 72% of subjects at 10 years (Branam 2022).

Osteochondral allograft transplantation can replace the whole osteochondral unit and be performed irrespective of the sizes of lesions and donor sites. It has been used successfully in the primary management of large OCDs. It transplants mature, viable hyaline cartilage to the defect area and matches the native surface anatomy (Haber et al. 2019). Recent advances have improved allograft availability and viability. However, disease transmission and immunogenicity remain potential complications associated with osteochondral allografts (Torrie et al. 2015).

Autologous chondrocyte implantation (ACI) is a regenerative/restorative intervention that provides relief for patients with significant OCDs. It involves the harvest of autologous chondrocytes, expansion of chondrocytes in a laboratory and implantation of chondrocytes into the damaged point, contributing to the development of hyaline cartilage (Migliorini et al. 2022a). In ACI's first and second generations, a periosteal flap or a collagen membrane was used to retain implanted chondrocytes at the defect sites. While in the third generation, the harvested chondrocytes were seeded and grew directly on a resorbable membrane that was then implanted in the damaged area (Migliorini et al. 2021, 2022b). Compared with microfracture, ACI exhibited improved outcomes, but there was no difference with osteochondral autograft transplantation (Harris et al. 2010).

16.3 Osteochondral Tissue Engineering Approaches for Articular Cartilage Regeneration

Current clinical treatment options for the osteochondral unit have limited success in keeping the structural and functional strengths of the regenerated tissues for an extended time. Fibrocartilage formation after microfracture and ACI impaired normal joint functions. Allografts might transmit unknown diseases. Innovative TE approaches are emerging for treating defects in the osteochondral unit, which combines scaffolds, cells and biomolecules for the regeneration of tissues. Various TE strategies have been introduced, such as injectable, customised, 3D-printed hydrogels/scaffolds, with and without cells, in vitro and in vivo evaluations. The primary design strategy was to mimic the native osteochondral structure and composition to induce zone-specific tissue regeneration. This section addressed the basic requirements of the osteochondral scaffolds, followed by the scaffold biomaterials for osteochondral regeneration. Then, the traditional and 3D printing TE approaches for osteochondral regeneration were summarised.

16.3.1 Requirement of Osteochondral Scaffolds

Numerous attempts have been made to construct scaffolds using various biomaterials by different methods to regenerate osteochondral units (Chocholata et al. 2019; Xu et al. 2022; Kimber and Shelton 2010; Lesage et al. 2022). There are several critical considerations for an appropriate osteochondral scaffold, comprising (1) good biocompatibility, bioactivity and surface topography to benefit the effectiveness of cell growth, (2) an appropriate biodegradability to ensure the formation of new tissues, (3) adequate mechanical strength to support load-bearing, (4) suitable architecture and porosity to ensure the interconnectivity and vascularity.

16.3.1.1 Biocompatibility, Bioactivity and Surface Topography

An ideal osteochondral scaffold should have appropriate biocompatibility to advance cell attachment, support cell growth and stimulate tissue regeneration. Surface topography and material properties can influence cell attachment, whilst the scaffold's microstructure influences cell adhesion, migration and differentiation (Tamaddon et al. 2018). The modification of surfaces topography, such as micro/nanostructures modification (Yang et al. 2019; Chen et al. 2019a; Xia et al. 2014), microroughness modification (Park et al. 2009; Gentile et al. 2010), patterned and structured surfaces modification (Papenburg et al. 2010; Kim et al. 2014; Qiu et al. 2014), significantly enhanced the bioactivity and biological reactions of materials and resulting in stimulated tissue reconstruction (Li et al. 2020). Improved bioactivity of the scaffold could result in enhanced osteoconduction (the ability to glow a new bone), osteointegration (strong integration between the scaffold and host tissue), osteoinduction (the ability to induce osteogenesis) and vascularisation (Turnbull et al. 2018; Albrektsson and Johansson 2001).

16.3.1.2 Biodegradability

Biodegradation is another concern for the osteochondral scaffold. The biodegradation property of the scaffold has to correspond to the formation speed of new tissue, and biodegradation's by-products do not be adverse to the human body and cause undesired immunological responses (Jeon et al. 2007; Anandhapadman et al. 2022). The implanted scaffold should remain firm for no less than 14–21 days to promote cartilage regeneration. Sufficient time is necessary to form structural compositions for subsequent tissue regeneration (Jeon et al. 2007). The biodegradability of the material must be considered prior to scaffold fabrication. For example, the *in vivo* degradation rate of biopolymers, such as collagen, was substantially different at different implantation sites and dependent on their enzymes' readiness and concentration (Nair and Laurencin 2007). At the same time, the hydrolytically degradable synthetic biopolymers had limited cross-site and subject-to-subject variability in comparison with enzymatically degradable biopolymers (Nair and Laurencin 2007; Katti et al. 2002). The intrinsic properties of the biopolymer determined its biodegradation rate (Ye et al. 1997). For instance, a volumetric PLGA scaffold degraded rapidly within 8 weeks upon introduction, with most of the builds losing

their shapes, followed by absorption after 16 weeks (Jeon et al. 2007), whilst a plain PLA scaffold was resorbed after 1.5 years.

16.3.1.3 Mechanical Strengths

The mechanical performance of the osteochondral scaffold is essential due to its load-bearing nature. The osteochondral scaffold is expected to correspond to the mechanical strengths of the original osteochondral unit and withstand the mechanical load. The Young's modulus of healthy human articular cartilage is between 5 and 25 MPa, subject to the areas of high or low weight-bearing measured, while compressive modulus varies from 2 to 10 MPa (Setton et al. 1999). Typically, cartilage experiences 3–10 MPa of stress in knee joints and up to 18 MPa in the hip joint (Elder and Athanasiou 2009). Regarding natural bones, the compressive modulus of cancellous bone has mid-range values of 90–400 MPa. In the transitional zone with intermediate stiffness between the articular cartilage and the subchondral bone, 'the modulus for the calcified cartilage was more than an order of magnitude lower than the underlying subchondral bone (Mente and Lewis 1994)'.

16.3.1.4 Architecture and Porosity

Scaffolds should have an intertwined porous structure with high porosity to support cell penetration, proliferation and differentiation to produce an appropriate environment for the cells (Loh and Choong 2013). The porous scaffold also benefitted from the diffusion of oxygen and nutrition, vasculature formation, network tissue formation and integration with the native tissues (Loh and Choong 2013; Liu et al. 2013). Pore sizes above 100 μm in scaffolds were preferred for osteoblasts in regenerating mineralised bone (Sugawara et al. 2005). Pore sizes between 90 and 325 μm were sufficient for promoting adequate vascularisation, cell migration and proliferation (Murphy et al. 2010; Lim et al. 2010). Human cortical bone (compact bone or dense bone) has a porosity of 5–15%, in contrast with the porosity of trabecular bone (cancellous bone or spongy bone) ranging from 40 to 95% (Morgan et al. 2018). It was suggested that a scaffold with a gradient porosity could be an appropriate alternative for natural bones (Abbasi et al. 2020).

16.3.2 Biomaterials for Osteochondral Regeneration

In consideration of the zonal compositions of the osteochondral unit, the materials must achieve the conditions of the regeneration of anisotropic functional and structural characteristics of the articular cartilage, the subchondral bone and the cartilage-bone connection. By comprehensive understanding and kind selection of biomaterials, composite scaffolds could combine their advantages to maximise the overall performance (Zhou et al. 2020; Zhang et al. 2021; Li et al. 2018).

16.3.2.1 Biomaterials for Cartilage Regeneration

Hyaline articular cartilage is composed of a dense extracellular matrix (ECM) with sparse distribution of chondrocytes. The ECM primarily consists of water, collagen

and proteoglycans, with extra non-collagenous proteins and glycoproteins (Sophia Fox et al. 2009). Various biomaterials have been applied to imitate the articular cartilage composition and physicochemical properties, including natural, synthetic and composite polymers (Armiento et al. 2018; O'Shea et al. 2022; Zhou et al. 2022; Yang et al. 2022). Biopolymers include polysaccharides (alginate, chitosan, hyaluronic acid (HA), glycosaminoglycans (GAGs) and cellulose), proteins (collagen, gelatin and silk fibroin) and bacterial-sourced polymers (hydroxy alkanates) (Vroman and Tighzert 2009). The biofunctional molecules contribute to bioactivity and tissue remodelling. For example, arginyl glycyl aspartic acid (RGD) sequence ligands on the surface of biopolymers were beneficial for cell interactions. They expedited cell migration, proliferation and differentiation with good biocompatibility, biofunctionality and biodegradability (Malafaya et al. 2007; Chen et al. 2011; Asghari et al. 2017). The drawbacks of biopolymers are their batch-to-batch variability and rather low mechanical property (Asghari et al. 2017).

The most frequently used synthetic polymers are aliphatic polyesters like polycaprolactone (PCL), and poly(lactic-co-glycolic acid) (PLG, PLGA) (Donnalaja et al. 2020). They have tunable stiffness, mechanical strength and degradation rate. They can be further tailored into different shapes and porosities to match the speed of cell migration or tissue formation (Asghari et al. 2017; Jeuken et al. 2016). However, the intrinsic hydrophobicity and lack of natural ligand binding sites made synthetic polymers exhibit poor bioactivity (Chen et al. 2011; Asghari et al. 2017; Jeuken et al. 2016). To overcome this, multi-component systems were designed to create composite scaffolds with improved performance (Yousefi et al. 2015). Surface functionalisation was carried out to promote cell behaviours, such as surface modification with chondroitin sulphate, ionised gases and alkaline (Amani et al. 2019), and incorporation of hydrophilic biomaterials or ECM proteins as constitutive elements of scaffold (Lu et al. 2011; Advincula et al. 2021). In addition, growth factors like transforming growth factors (TGF- β) and bone morphogenetic proteins (BMP) were grafted onto these polymers to facilitate cell proliferation and differentiation and tissue repair and regeneration (Zhou et al. 2017).

16.3.2.2 Biomaterials for Subchondral Bone Regeneration

When designing subchondral bone scaffolds, biomechanical properties, appropriate new bone in-growth and incorporation with surrounding tissues should be considered. The most common biomaterials include calcium phosphates (CaPs), such as monophasic hydroxyapatite (HAp), tricalcium phosphate (TCP), biphasic calcium phosphates and multiphasic bioglasses (BGs), as well as metallic materials (Amjad et al. 1978). CaPs have physicochemical similarities to the biological apatite in human bones, making them suitable candidates for subchondral bone repair. Their compositions could be customised to control biodegradation rate, processability and mechanical properties to match bone formation (Porter et al. 2009). They could also be used alone or combined with other materials (Zhou et al. 2020). CaPs were the most favourable biomaterials used for bone regeneration (Lesage et al. 2022). BGs with excellent bioactivity are usually classified into silicate, phosphate, or borate-based glasses. The release of cations, the formation of the hydroxycarbonate apatite

layer in physiological fluids and the chondrogenesis ability attracted interest in osteochondral bone regeneration (Rahaman et al. 2011).

Metallic materials such as titanium (Ti) and its alloys, stainless steel and cobalt-chrome alloys were commonly used as orthopaedic implants for subchondral bone due to their capability of withstanding high mechanical loading (Love 2017). Despite adequate mechanical strength, most metallic materials are inert. As permanent metals, they are not degradable and form wear particles over time. Bioceramics could be coated on metallic materials to stimulate apatite formation and further integrate the implant with subchondral bone (Yi et al. 2014; Coathup et al. 2017; Mohammadi and Sepantafar 2016). Magnesium (Mg)-based alloys have emerged as new metallic materials due to their excellent bioactive, biodegradable and osteopromotive properties (Yang et al. 2017). However, the inappropriate in vivo degradation rate and unsatisfactory corrosion resistance restricted their capability to provide adequate mechanical strengths and reduced biocompatibility with hydrogenation (Zhou et al. 2021; Deng et al. 2019a). To overcome the constraints, an Mg/Ti hybrid fixation system was designed to ensure adequate mechanical strengths and promote fracture healing by enhancing callus tissue formation (Tian et al. 2018).

16.3.3 Traditional TE Approaches

To distinguish 3D printing technology, the terminology ‘traditional TE approaches’ is mentioned in this section includes but not limited to cast mould/gelation (Amann et al. 2021; Shang et al. 2020; Madry et al. 2020; Lee et al. 2012; Zhu et al. 2019), freeze-drying (Amann et al. 2021; Browe et al. 2022; Ye et al. 2022), cryogelation (Nikhil and Kumar 2022), etc. Traditional TE approaches for osteochondral regeneration were reviewed based on cell-free and cell-seeding, cell-based and scaffold-free approaches.

16.3.3.1 Cell-Free and Cell-Seeding TE Approaches

Generally, a one-stage cell-free scaffold was employed to handle full-thickness osteochondral lesions in the knee. The layered scaffold mimicked the native composition and structure of the osteochondral unit. The cartilage-like collagen layer was on the top with a smooth surface, and the rough bone-like HAp/collagen layer was at the bottom. The layered scaffold was press-fit into the defect area to induce cells towards an ordered regeneration of both cartilage and bone tissues. Promising outcomes were observed in animal models and subsequently in clinical cases. In comparison with cell-based regenerative methods, the advantages of this approach were a one-stage surgical procedure, extensive lesions, off-the-shelf availability and lower costs (Sessa et al. 2019). The same cell-free scaffolds were implanted in the whole range of 54 patients for a minimum follow-up of 5 years. Substantial clinical benefits were found and maintained over 5 years regardless of gender, age, or lesion aetiology. The best results were achieved in patients below 35 years old or

influenced by OCDs. The biomimetic scaffold was further proved to be a feasible solution for treating osteochondral lesions (Ricci et al. 2021).

A multilayered chitosan-collagen-octacalcium phosphate (OCP) scaffold was developed using biomaterial gradients to mimic the native osteochondral transition. Collagen concentration was decreased while the amount of OCP was increased towards the bony layer. The scaffold showed good integration and mechanical stability. Highly interconnected pores ranging from 75 to 240 μm supported cell distribution and interactions. The porous structure and higher content of collagen in the cartilage layer induced chondrogenesis. By adding OCP to the bony layer, the scaffold stimulated osteogenic induction with a concurrent expression of markers for differentiated and hypertrophic chondrocyte-like cells. A zone-specific gene expression was shown with upregulated COL2A1 in the cartilage zone and upregulated RUNX2, COL1A1, BGLAP and SPP1 in the bony layer. A steady increase of Ca and P over time in the bony area indicated mineralisation. The gradient and multiphasic scaffold might be promising for osteochondral unit repair (Amann et al. 2021).

A bilayered cryogel scaffold was designed and fabricated with chitosan-gelatin-chondroitin sulphate (CGC) as the top layer and nano-hydroxyapatite-gelatin (HG) as the bottom layer to imitate cartilage and subchondral bone by cryogelation without delamination of the layers. The mixture of 1% chitosan and 5% gelatin for CGC cryogel exhibited better elastic and mechanical stability. Highly porous CGC cryogel supported the uptake of synovial fluid and the transportation of nutrients to chondrocytes in synovial joints. Less porous HG cryogel compared with CGC and a high amount of nHAp particles embedded into the pore walls provided an osteoconductive environment for cell growth. The swelling ratios of CGC and HG cryogel were 23 ± 0.5 and 6 ± 0.3 , respectively. They retained their properties during repeated swelling and de-swelling cycles.

Moreover, the cryogel scaffold was not deformed under high dynamic load. The swelling kinetics and mechanical stability made the cryogel scaffold suitable for the osteochondral unit as the load-bearing region. Goat chondrocytes on CGC cryogel showed significant proliferation and good biocompatibility. The increased ALP level in pre-osteoblastic cells cultured over HG cryogel showed the stimulated differentiation towards osteoblast. Furthermore, CGC cryogel delivered 80% of exosomes at the defect site in 72 h and released 18.7 μg cryogel extract with a chondroprotective effect. It was expected that the combination of polymeric cryogel with chondrocyte exosomes was promising for the treatment of OCDs (Nikhil and Kumar 2022).

Similarly, a bilayered structure with nanotextured silk fibroin (SF)-chondroitin sulphate (CS)/HAp nanowire (SF-CS/HAp in short) was fabricated for osteochondral repair. The permeation of glutinous SF-CS solution to HAp nanowires made the bilayers inseparable from the continuous and gradual transition at the cross-section region. The tensile (~ 5.26 MPa) and compressive modulus (~ 6.7 MPa) of the SF-CS/HAp membrane were comparable to that of natural cartilage tissue. The nanotopography, moderate elasticity and continuous discharge of CS from the SF-CS layer upregulated the chondrogenic gene expression (COL2A1, ACAN and SOX-9) of bone marrow mesenchymal stem cells (BMSCs). Meanwhile, the HAp layer facilitated osteogenic differentiation of

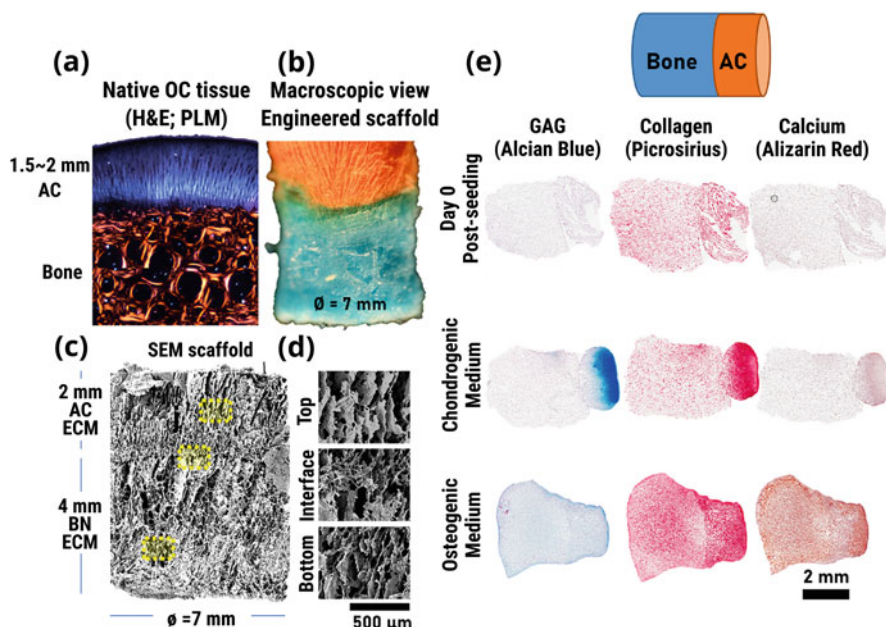


Fig. 16.2 Spatial differentiation of MSCs on a bilayered ECM-derived scaffold. (a) Hematoxylin and eosin (H & E) staining of native osteochondral goat tissue. (b) Macroscopic view of the ECM-derived scaffold. (c, d). Scanning electron microscopy (SEM) images of the bi-layered scaffold. (e). Histological images before and after culturing in chondrogenic and osteogenic medium for 28 days. Reproduced under the terms of the CC-BY license (Browe et al. 2022). Copyright 2022, the Authors. Published by Elsevier

BMSCs with substantially strengthened protein and gene levels of OCN and OPN. Twelve weeks following implantation *in vivo*, new tissues were formed and integrated with surrounding normal cartilage in SF-CS/HAp group. Micro-CT images showed a higher degree of bone healing and increased newly growing calcified tissues in the bony area in SF-CS/HAp one. The biomimetic SF-CS/HAp membrane has the potential for osteochondral regeneration (Shang et al. 2020).

Furthermore, to replicate the hierarchical structure of native articular cartilage (Fig. 16.2a), Daniel J. Kelly's group developed bilayered ECM-derived scaffolds with solubilised bone-ECM as the bone layer and solubilised cartilage-ECM as the cartilage layer (Fig. 16.2b). The freeze-drying process was modified with controlled heat transfer, and additional annealing step to fabricate anisotropic scaffolds with bigger holes, resulting in homogenous cellular infiltration, increased sulphated GAG deposition and more cartilage-like collagen network both *in vitro* and *in vivo*. Under chondrogenic and osteogenic culture conditions, the bilayered scaffold demonstrated zone-specific differentiation of MSCs. Intense staining with alcian blue and picrosirius red in the cartilage layer showed hyaline-like cartilage matrix sGAG and collagen deposition. Collagen and calcium deposition were observed in both

bone and cartilage layers (Fig. 16.2e). The capability of guiding neo-tissue organisation and recapitulation of zonal structures of native cartilage made the ECM-derived scaffold an option for osteochondral repair (Browe et al. 2022).

There is an emerging trend for targeted repair of the osteochondral unit that gene vectors were delivered precisely and invasively to reduce intra-articular vector spread and potential loss of therapeutic genes. A recombinant adeno-associated virus (rAAV) vector was control-released by an injected and thermosensitive hydrogel based on polyethylene oxide (PEO)–polypropylene oxide (PPO)–PEO poloxamers. The effect of in situ discharge of an rAAV encoding for the chondrogenic sex-determining region Y-type high-mobility group box 9 transcription factor (SOX-9) on full-thickness OCDs was evaluated in the Göttingen minipig model in vivo. After 4 weeks, SOX-9/hydrogel filled the defects and increased the thickness of the cartilage compared with other groups. More collagen type II (COL II) deposition in the SOX-9/hydrogel defects with visually better collagen distribution towards the subchondral bone indicated a more analogous approximation of normal cartilage, and in situ chondrogenesis was significantly improved by SOX-9/hydrogel. Meanwhile, SOX-9/hydrogel protected the subchondral bone plate from early bone loss. The copolymers are promising for rAAV delivery in vivo to support cartilage repair (Madry et al. 2020).

Lack of effective chondrogenic-inducing factors (e.g. TGF- β family) restrict the self-regeneration of cartilage. However, the inaccessibility and instability of TGF- β restrict its use in the treatment of OCDs. It was proposed to load oligopeptide LIANAK (CM) into self-assembling RAD to achieve a TGF- β 1-simulating peptide (RAD-CM). Then, RAD-CM and decellularised cartilage extracellular matrix (DCM) were combined to form a synthesised scaffold (R/C/D) that demonstrated excellent bioactivity and structural stability. The in vivo studies showed that the neo-tissue in the R/C/D areas demonstrated the typical morphology of hyaline cartilage with flat and smooth, better integration with surrounding normal cartilage after 16 weeks (Fig. 16.3a). The R/C/D group scored the best among all the groups in the International Cartilage Repair Society (ICRS) scoring of overall repair assessment (Fig. 16.3b). After 8 and 16 weeks, the highest deposition of GAGs in the R/C/D group demonstrated the strongest cartilage matrix secretion (Fig. 16.3c). Safranin O/fast green (SOG) staining demonstrated the most appropriate osteochondral interface and subchondral bone matrix in the R/C/D scaffold with continuous and smooth deposition of collagen I. R/C/D scaffold promoted neocartilage restoration and the regeneration of the osteochondral unit (Ye et al. 2022).

Treating large osteochondral lesions is the most challenging, especially with signs of OA. Massive allograft or unicompartmental replacement was often needed for sizeable condylar defect replacement. A hemicondylar aragonite-based scaffold (size: $19 \times 8 \times 8$ mm) was proposed to reconstruct large osteochondral lesions of the medial femoral condyle using a goat model for a period of 12 months. No persistent inflammation and no evidence of OA improvement was observed among all goats. The scaffold was degenerated and substituted by trabecular bone tissue, and an overlying hyaline cartilage surface was seen to be well-developed with the surrounding tissue. It showed concurrent regeneration of the osteochondral tissues of both the cartilage and the subchondral bone (Kon et al. 2020).

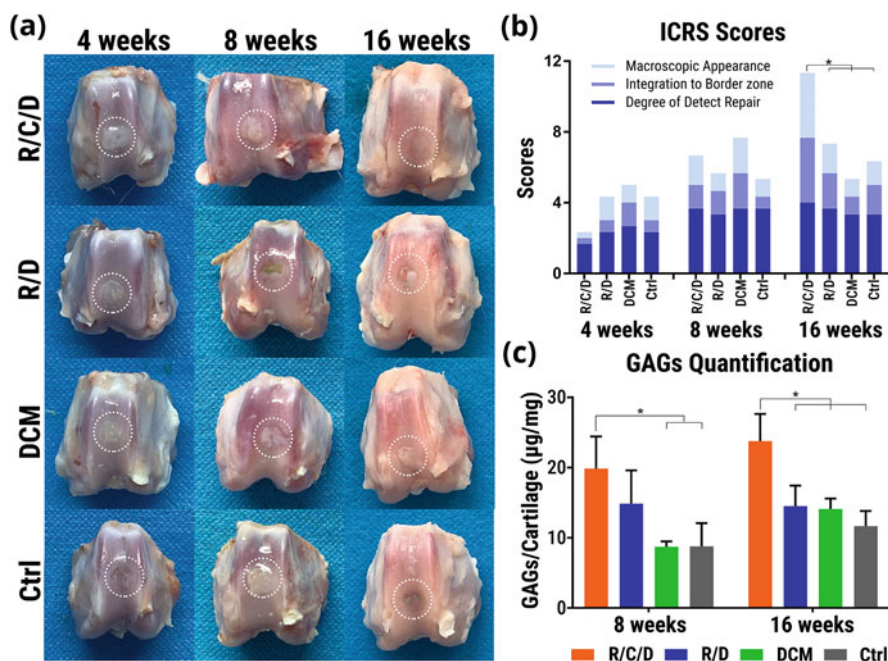


Fig. 16.3 In vivo evaluation of rabbit knee samples. (a) Macroscopic images of the newly formed tissues at defect areas. (b) ICRS scores of neocartilage. (c) GAGs quantification of neocartilage. Reproduced with permission (Ye et al. 2022). Copyright 2022, Elsevier

16.3.3.2 Cell-Based TE Approaches

The cell-based approaches have obtained increasing attention since being introduced in 1987. MSCs have been widely used in cartilage engineering in recent years. On the one hand, it is not easy to harvest sufficient autologous chondrocytes, and they tend to lose their phenotype during in vitro expansion (Von Der Mark et al. 1977; Kisiday 2019). On the other hand, autologous hMSCs have shown comparable or more remarkable improvement than chondrocytes for cartilage repair (Nejadnik et al. 2010). Moreover, the well-established protocols of chondrogenic differentiation of hMSCs made the application of cell-based therapies realistic in clinical trials (Somoza et al. 2014; Wei et al. 2013; Seo and Na 2011). The biodegradable polymers were applied as substitute scaffolds for the in vitro growth of living cells and implantation to the defects (Gomoll et al. 2010). Numerous attempts have been made to achieve osteochondral regeneration by forming the hyaline cartilage and the subchondral bone, which should be mechanical, functional, stable and integrated with surrounding native tissues.

Injectable hydrogel-based scaffolds could provide the chondrogenic/osteogenic environment of MSCs and enhance the transportation of nutrients to encapsulated cells. Synovium-derived mesenchymal stem cells (SDSCs) were embedded into

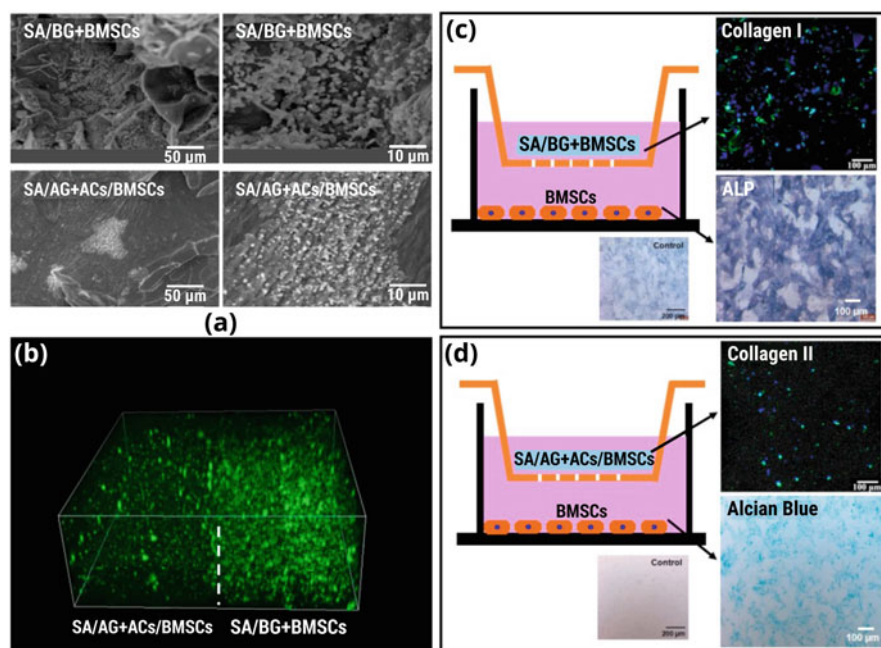


Fig. 16.4 (a) SEM images of the SA/BG and SA/AG hydrogels with encapsulated cells. (b) Live-dead staining of BMSCs and ACs/BMSCs in SA/BG and SA/AG hydrogels on day 7. (c) Immunofluorescence staining of COL I and ALP of BMSCs inside and outside the SA/BG + BMSCs hydrogels. (d) Immunofluorescence staining of COL II of ACs/BMSCs inside and alcian blue staining of BMSCs outside the SA/AG + ACs/BMSCs hydrogels. Reproduced with permission (Zhu et al. 2019). Copyright 2019, Elsevier

collagen/hyaluronic acid/fibrinogen (COL/HA/FG) composite hydrogel. Highly viable SDSCs in the composite hydrogel expressed COL II, aggrecan and SOX-9. When applied to a rabbit model with OCDs in the knee, hyaline cartilage-like tissue was produced after 24 weeks (Lee et al. 2012).

Furthermore, a two-layered injectable scaffold was produced for osteochondral repair with a thermosensitive SA/agarose (AG) hydrogel containing BMSCs and articular chondrocytes (ACs) (SA/AG + ACs/BMSCs) for articular cartilage regeneration, and a BMSCs encapsulated sodium alginate (SA)/BG hydrogel (SA/BG + BMSCs) for subchondral bone regeneration. The continuous SA in the stratified structure of scaffolds mimicked the native osteochondral unit without a clear interface between the two layers. BMSCs were consistently distributed on the pore wall of the SA/BG hydrogels, while BMSCs and ACs were aggregated within the pores of SA/AG hydrogels (Fig. 16.4a). Figure 16.4b showed that most cells were alive after encapsulation in the construct for 7 days. After 10 days, higher collagen I (COL I) expression in BMSCs within SA/BG + BMSCs groups and ALP expression in BMSCs cultured on the same well under the Transwell (Fig. 16.4c) than that in control confirmed the stimulated osteogenic differentiation of BMSCs by

the BG. Alcian blue staining in Fig. 16.4d demonstrated the stimulated chondrogenic differentiation of BMSCs. Moreover, the co-culture of ACs and BMSCs within the builds induced the chondrogenic differentiation by the expression of the COL II. Twelve weeks after the injection of the hydrogel into OCDs of the rats, the combination of SA/BG + BMSCs and SA/AG + ACs/BMSCs constructs demonstrated the best cartilage and subchondral bone regenerations among all groups. The fresh grown tissues were comparable to native ones while integrating better with surrounding normal tissues (Zhu et al. 2019).

A compartmented implantable medical device containing a triple-3D structure was developed to activate osteochondral differentiation from hMSCs for osteochondral regeneration. hMSCs were cultivated as 3D cell microtissues (MTs) to imitate natural mesenchymal condensation during endochondral differentiation. hMSCs MTs were seeded on Bio-Gide® collagen membrane as compartment 1 for subchondral bone engineering. The significantly increased expression of RUNX2, OCN and BSP-II from day 7 to day 28 demonstrated the osteogenic differentiation of hMSCs and subsequent mineralisation *in vitro*. hMSCs MTs-encapsulated alginate/HA hydrogel (compartment 2) were grown in a chondrogenic medium for cartilage engineering. Immunostaining and histological staining of cartilage tissue markers of SOX-9, COL II, aggrecan and GAGs confirmed the chondrogenic differentiation of hMSCs. The combination of both compartments resulted in a hybrid implant with an osteogenic/chondrogenic gradient in the physiological osteochondral unit (Keller et al. 2015).

16.3.3.3 Scaffold-Free TE Approaches

Although various scaffold-based TE approaches have been developed and *in vitro* cell culture studies were promising, long-term outcomes were still uncertain and limited translation from bench to bedside was achieved. Scaffold-free TE approaches were also developed for osteochondral regeneration. A range of cells have been investigated for osteochondral regeneration, like BMSCs (Itokazu et al. 2016; Beane and Darling 2012; Lee et al. 2018), human periosteum-derived progenitor cells (hPDCs) and human articular chondrocytes (hACs) (Mendes et al. 2020), induced pluripotent stem cells (iPSCs) (Weil et al. 2012; Teramura et al. 2010), etc. Cell pellets or MTs with high density were prepared to enhance cell-cell interactions, mimic embryonic development and produce native ECM.

Owing to differentiation capabilities into osteoblasts and chondrocytes under specific conditions, BMSCs were commonly applied to cartilage TE (Beane and Darling 2012). The culture conditions were optimised to generate stable and reliable scaffold-free cartilage-like cell sheets from hBMSCs. The inclusion of fibroblast growth factor 2 (FGF-2) during the expansion of hBMSCs not only improved cell proliferation and chondrogenic differentiation but also reduced the concentration of fetal bovine serum (FBS). The hBMSCs created cell sheets were transplanted into the OCDs in nude rats for up to 12 weeks. The Wakitani scores of the cell-sheets groups were significantly higher than those of the control groups, demonstrating the positive effect of cell sheets on osteochondral regeneration (Itokazu et al. 2016).

However, the mechanical stability of cell sheets was a concern that might cause implantation failure. A cellular agglomerates-based strategy was employed to adequately anchorage scaffold-free cells into the defect site. Aggregated spherical hBMSCs were prepared and transplanted into the OCDs of the rabbit model with fibrin glue. The superior overall osteochondral restoration was observed in the spherical hBMSCs group compared with single-cell suspension and control groups after 12 weeks. However, long-term evaluation may be necessary to monitor delayed immune response and cartilage regeneration (Lee et al. 2018).

Since immature osteochondral grafts (OCGs) could integrate with surrounding tissues and regenerate osteochondral units, biomimetic and scaffold-free TE constructs (bTECs) were engineered to mimic the morphology and structure of immature OCGs for the restoration of deep OCDs. The micromasses of hPDCs and hACs were stacked together to form bTECs and implanted into an OCD (1.6 mm in diameter, 1.6 mm in depth). After 4 weeks, a cartilage layer was presented with Safranin O positivity, and a new bone layer was formed underneath with a layer of non-mineralised and non-cartilaginous tissue in between (Fig. 16.5a). Sixteen weeks later, high GAG content was maintained in the cartilage layer, and more newly formed bone tissues were observed. The significantly decreased tissue layer between the cartilage and bone layers indicated an active tissue regeneration from week 4 to week 16 (Fig. 16.5b). Homogeneous COL II deposition after 4 weeks (Fig. 16.5c) and gradient deposition from top to bottom after 16 weeks (Fig. 16.5d) showed the zonal distribution in the articular cartilage. COL I was prominent in the GAG-negative tissue layer after 4 weeks (Fig. 16.5e), while it was mainly identified at the interface between the cartilage and the new bone after 16 weeks (Fig. 16.5f). Hierarchically organised cell-based TE constructs mimicking immature OCGs was an attractive strategy for treating deep OCDs (Mendes et al. 2020).

16.3.4 3D-Printed Osteochondral Scaffolds for Articular Cartilage Regeneration

To successfully fabricate an appropriate osteochondral scaffold for articular cartilage regeneration, the hierarchical architecture is a vital factor besides the compositions. With extensive comprehension of the biological sciences of the osteochondral unit, 3D printing technology has become an emerging TE approach for the fabrication of osteochondral scaffolds. The application of 3D-printed osteochondral scaffolds was reviewed from monolayer scaffold-based strategies to biphasic, multiphasic and even gradient scaffolds over the past few years.

16.3.4.1 Monophasic Scaffolds

Monophasic scaffolds represented the earliest standard technique of scaffold fabrication, relying on homogeneous architecture (structural porosity) and composition (materials, cell types and bioactive factors) system to repair the entire osteochondral unit. Deng et al. 3D printed a monophasic $\text{L}_2\text{C}_4\text{S}_4$ bioceramic scaffold (Fig. 16.6). The scaffold showed excellent dual bioactivities (chondrogenesis and osteogenesis),

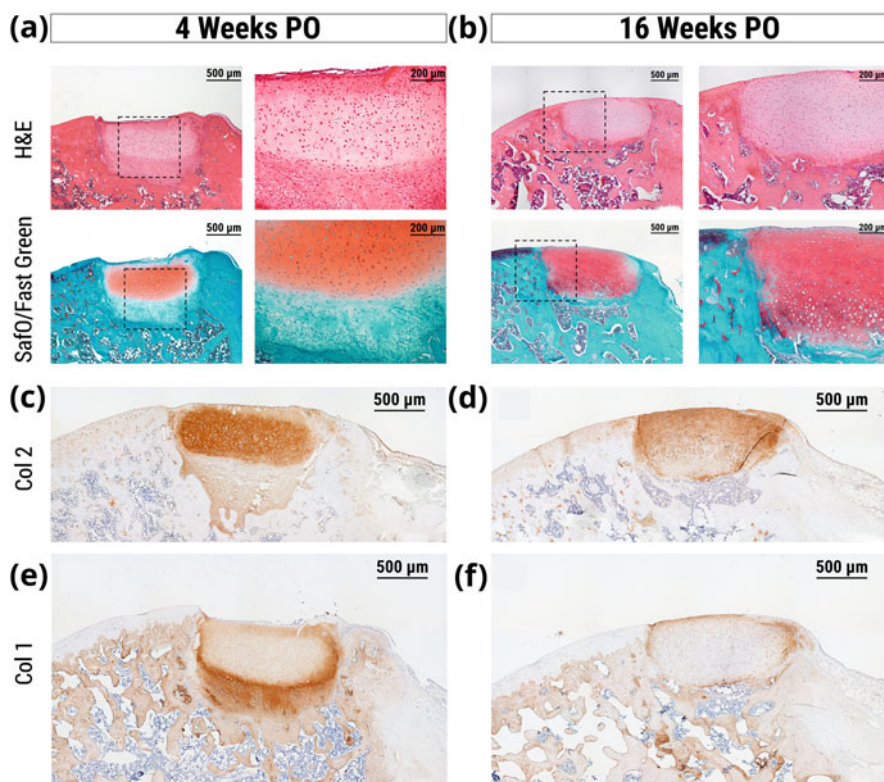


Fig. 16.5 Histological evaluation of the osteochondral regeneration. (a) The cartilage layer maintained its Safranin O positivity at 4 weeks postoperatively (PO) and 16 weeks PO (b). (c) COL II immunostaining 4 weeks PO and 16 weeks PO (d). (e) COL I immunostaining 4 weeks PO and 16 weeks PO (f). Reproduced with permission (Mendes et al. 2020). Copyright 2020, Elsevier

while its excellent mechanical properties and good degradability could support subchondral bone regeneration. Furthermore, there was no acute or chronic toxicity due to lithium ions release within the 12 weeks in vivo. Besides, the deposition of chondrocytes and MSCs into this $L_2C_4S_4$ bioceramic scaffold resulted in the construction of cartilage and subchondral bone with different cellular and ECM composition (Chen et al. 2019b). To avoid the delamination of two layers and offer a stable and hierarchical structure for osteochondral regeneration, researchers proposed to enhance chondrogenesis or osteogenesis by surface modifications further. The combination of 3D printing technology and hydrothermal method successfully created micro/nanostructured surfaces on the bregidite scaffolds. It provided an alternative strategy to enable dual-lineage bioactivity in osteochondral regeneration using surface microarchitecture to construct multiple tissues (Deng et al. 2019b). To solve the problem caused by excess reactive oxygen species (ROS) in OCDs, which usually results in OA and cartilage degeneration. A 3D-printed novel antioxidant

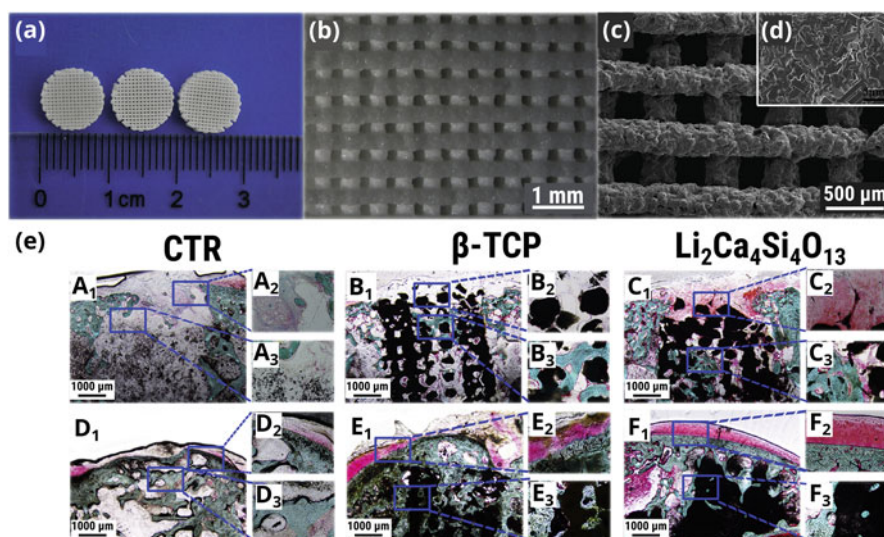


Fig. 16.6 Morphology and in vivo regeneration quality of 3D-printed monophasic $\text{Li}_2\text{Ca}_4\text{Si}_4\text{O}_{13}$ bioceramic scaffolds. (a) Digital photograph. (b) Optical microscope image. (c and d) SEM images. (e) Cartilage and subchondral bone regeneration in vivo after implantation for 8 and 12 weeks. Safranin-O/fast green staining at week 8 (A_1 – C_3) and week 12 (D_1 – F_3) after implantation. The black colour, blue colour and red colour represent scaffold, bone and cartilage, respectively. S-O staining showed the largest volume of GAGs in $\text{Li}_2\text{Ca}_4\text{Si}_4\text{O}_{13}$ group after 12 weeks. Reproduced with permission (Chen et al. 2019b). Copyright 2019, Elsevier

akermanite (AKT) and hair-derived nanoparticles/microparticles (HNPs/HMPs) bioceramic scaffold was developed for ROS depletion and osteochondral tissue regeneration. Results demonstrated no inflammatory reaction at 6 and 12 weeks after implantation of HNP-AKT scaffolds. HNPs/HMPs scaffolds stimulated osteogenic differentiation of rBMSCs through the glucose transporter pathway. Numerous GAGs and hyaline cartilage were well-developed with the surrounding cartilage. The HNP-AKT scaffolds facilitated osteochondral tissue regeneration by alleviating oxidative stress and promoting stem cell differentiation (Deng et al. 2022).

16.3.4.2 Biphasic and Multiphasic Scaffolds

There were some limitations in enhancing the site-specific cell differentiation and matrix deposition as the distinctions in tissue composition and structure from articular cartilage to subchondral bone. It was pretty challenging to regenerate distinct tissues in an OCD by a monophasic scaffold. Various biphasic and multiphasic scaffolds have been produced using two or three different materials, structures, or compositions to fabricate bilayered or multilayered constructs. Polymers and hydrogels were suitable for soft cartilaginous regions and stiff matrices for the subchondral bone layer. The opposing regions were combined as an osteochondral construct with distinct structural and mechanical properties.

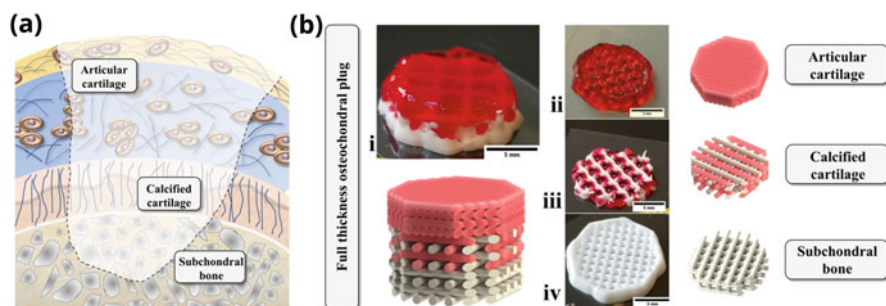


Fig. 16.7 Multi-channel 3D printing of biphasic scaffolds for osteochondral regeneration. (a) Illustration of a zone-specific hierarchical structure of osteochondral unit. (b) 3D plotting of artificial full-thickness osteochondral scaffold. (i) A combination of cell-laden hydrogel and partly mineralised calcium phosphate cement (CPC)-supported regions resembling (ii) articular cartilage and (iii) intermediate calcified cartilage and (iv) subchondral bone (pure CPC). Reproduced under the terms of the CC-BY license (Kilian et al. 2020). Copyright 2020, the Authors. Published by Springer Nature

By combining multi-channel plotting and extrusion-based 3D printing approaches, Kilian et al. provided a solution for generating heterogeneous osteochondral grafts with volumetric mineralised constructs and osteochondral tissue substitutes (Fig. 16.7). A biphasic bioceramic scaffold was extrusion printed with two different materials. The subchondral bone was made of acellular ceramic paste, while the cartilaginous phase was from a cellular alginate-based hydrogel. By modifying the Ca and P concentrations of two materials, a calcified cartilage phase with similar mineral content inside the osteochondral interface has been resembled. After 3 weeks of *in vitro* culture, alginate-methylcellulose-encapsulated chondrocytes were differentiated into respective lineages and secreted sufficient ECM components (Kilian et al. 2020). This study illustrated the interaction between mineralisation and chondrogenesis, an alternative possibility for osteochondral regeneration.

A human turbinate-derived mesenchymal stromal cells encapsulated multiphasic scaffold was bioprinted with hydrogel composed of HA, pepsin-treated collagen (atelocollagen) and stimulative components for articular cartilage regeneration (Shim et al. 2016). A mechanically stable host-guest chemistry-based supramolecular hydrogel strategy was employed to construct two ECM hydrogels without using harmful crosslinkers. HA along with TGF- β constituted to cartilage region, and atelocollagen combined with BMP-2 contributed to the formation of the bone layer. *In vivo* studies showed that thick neocartilage tissue was formed at the centre of the OCD of rabbits after 8 weeks of implantation. This implanted construct did not elicit any observable inflammatory responses *in vivo*.

Besides continuous corrosion and injury, chronic inflammation in the joints deteriorates the progression of OCDs. A 3D multilayered scaffold was printed with the encapsulation of BMSCs to overcome the problem, in which β -TCP incorporated PCL layer for bone reconstruction and KGN-grafted

PCL/methacrylated HA (MeHA) layer for cartilage regeneration (Liu et al. 2021). The BMSCs-laden scaffold stimulated the osteochondral regeneration efficiently by promoting the deposition of ECM protein (COL I) and preventing the generation of interleukin-1 β . An anti-inflammatory layer of diclofenac sodium (DC) containing MeHA was on the exterior of the build to refrain from the inflammatory microenvironment.

16.3.4.3 Gradient Scaffolds

The osteochondral unit is a zonal structure with gradient-based biological and mechanical properties. TE approaches involve the fabrication of osteochondral scaffold to replicate the strengths of musculoskeletal and other heterogeneous tissues (Bittner et al. 2018). Gradient designs can be categorised into a discrete gradient (i.e. a biphasic or multiphasic) and a continuous gradient form. The fixation between different interfaces of biphasic or multiphasic scaffolds might not be stable in vivo and thus could not mimic the native interface of osteochondral tissue (Xu et al. 2022). A continuous gradient scaffold resembles the natural osteochondral unit more closely without a distinct interface between adjacent layers (Zhang et al. 2020).

For physical gradient scaffolds, structural and biomechanical features may be altered along the axis to simulate the transformations from cartilaginous to calcified zone and then subchondral bone. Controlled stiffness of the local environment strongly affected cell behaviours (Wu et al. 2017; Singh et al. 2010). MSCs expressed higher osteogenic markers when cultured on stiff (15–40 kPa) versus soft (0.1–1 kPa) matrices (Engler et al. 2006). The porosities, pore sizes and shapes of the scaffolds could be adjusted in gradient patterns to improve site-specific cell differentiation. Di Luca et al. revealed that chondrogenesis of hMSCs in gradient scaffolds was significantly improved with decreasing pore sizes (Di Luca et al. 2016a), while osteogenesis favoured larger pore sizes (Di Luca et al. 2016b). 3D-printed PCL scaffolds with gradient square pore shapes mainly facilitated the chondrogenesis of stem cells, and rhomboidal pores enhanced the osteogenesis of cells in vitro (Di Luca et al. 2016c).

The gradient component is another strategy. A variety of osteo- and chondro-inductive materials were used to form scaffolds with gradient compositions, significantly stimulating the proliferation and differentiation of relative cells. Selective laser sintering (SLS)-derived HA gradient scaffolds and biohybrid gradient scaffolds have demonstrated favourable biocompatibility in vitro, and thus their composition gradient designs could boost the early subchondral bone repairing and the development of hyaline cartilage. The regenerated cartilage and subchondral bone tissues were combined with nearby tissues well (Du et al. 2017). Gao et al. 3D printed reinforced biohybrid gradient hydrogel scaffolds for treating OCD. This hydrogel matrix consisted of cleavable poly(N-acryloyl 2-glycine) (PACG) and methacrylated gelatin (GelMA) (PACG-GelMA), with tunable biodegradability by adjusting the ACG/GelMA ratios. Bioactive manganese ions (Mn^{2+}) were inserted into the top cartilage layer of the gradient PACG-GelMA hydrogel scaffold to boost osteochondral tissue repair. Meanwhile, the bioactive glass (BG) was integrated into the bottom subchondral bone layer of the scaffold to enhance new bone

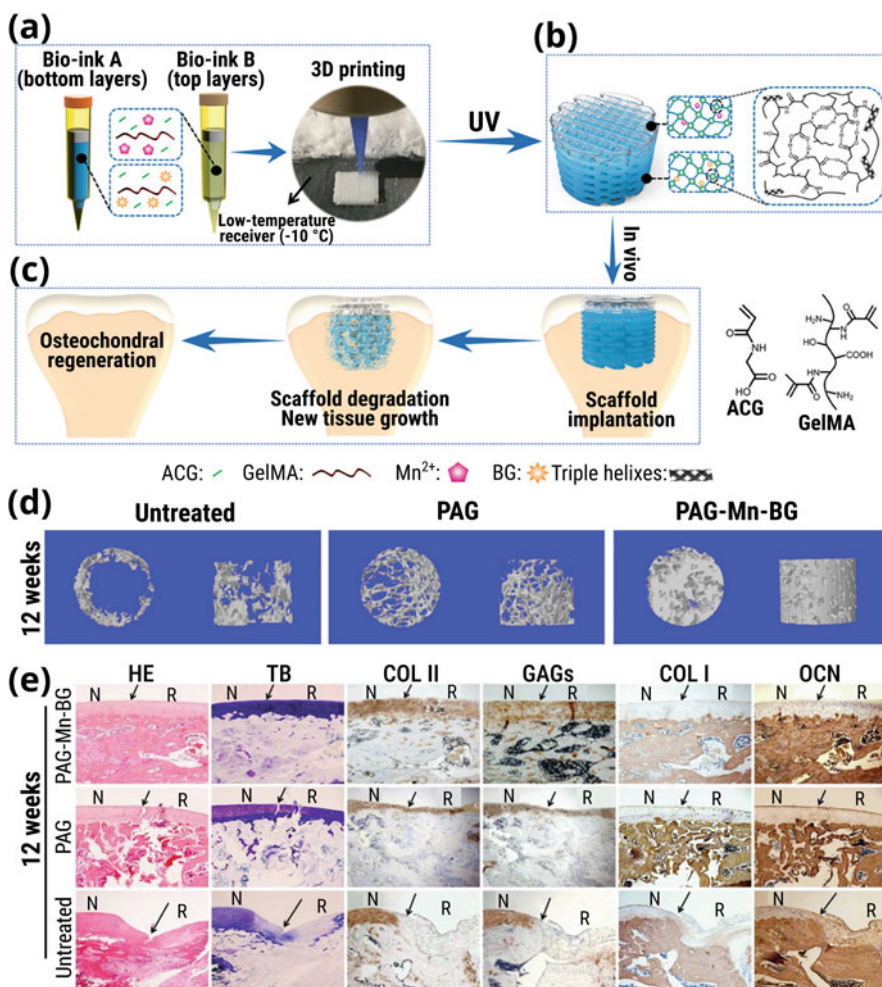


Fig. 16.8 3D printing and in vivo implantation of the biohybrid gradient scaffolds for osteochondral TE. **(a)** Schematic illustration of 3D printing of biohybrid scaffolds with the assistance of a low-temperature receiver. **(b)** UV light-initiated polymerisation of the hydrogel. **(c)** Schematic illustration of in vivo osteochondral regeneration by scaffold implantation. **(d)** Micro-CT images of the repaired subchondral bone after 12 weeks. **(e)** Histological analysis of the implants after 12 weeks. (N normal cartilage; R repair cartilage; the arrows indicate the margins of the normal cartilage and repaired cartilage; Scale bar = 200 μm). Reproduced under the terms of the CC-BY license (Gao et al. 2019). Copyright 2020, the Authors. Published by WILEY-VCH Verlag GmbH & Co.

regeneration. Results demonstrated, after 12 weeks post-implantation, the formation of new cartilage with similar thickness to the adjacent tissue and new bone filled in the subchondral bone area in the PAG-Mn-BG scaffold group (Fig. 16.8) (Gao et al. 2019). Another high-strength PNT hydrogel biohybrid gradient was produced for

similar osteochondral repair purposes using thermal-assisted extrusion 3D printing. The β -TCP in the subchondral bone phase enhanced the stiffness and osteoinductivity of the printed scaffold, and the TGF- β 1 in the cartilage phase stimulated chondrogenic differentiation (Gao et al. 2018). The 3D-printed scaffold with a high fidelity maintained highly interconnected porosity, desired mechanical performance and good biocompatibility. It offered a promising option for osteochondral regeneration.

16.4 Concluding Remarks

Current non-surgical and surgical treatments for OCDs are unsatisfactory and encounter many limitations, difficulties and risks. If only the cartilage surface is repaired without addressing the subchondral bone, joint pain may remain, and OCD could appear again. Further development of more effective, targeted therapeutic approaches is necessary to repair and regenerate the osteochondral unit. Traditional TE approaches and 3D printing strategies in this field have progressed sustainably during past decades. Along with the in-depth understanding of the complex composition and hierarchical architectures of the osteochondral unit, researchers tended to fabricate layered and/or gradient scaffolds with compositional or structural gradients mimicking the native osteochondral unit. Hydrogel systems and specified architectures provided a favourable environment for cell growth and tissue formation. The capability of MSCs to differentiate into osteoblasts or chondrocytes made them abundant cell sources. Growth factors further induced osteogenic or chondrogenic differentiation of MSCs. In vitro cell culture studies and short-term in vivo animal models have shown promising outcomes in the regeneration of osteochondral unit. Nevertheless, the long-term durability is still unclear. Large animal models are preferred to evaluate the function or pathology of osteochondral regeneration for more than 1 year period. Currently, only limited preclinical and clinical studies have been conducted. More preclinical and clinical case studies with newly fabricated 3D TE scaffolds are expected within years. It is suggested to consider good manufacturing practices and regulatory policies as well during the design and development of innovative TE scaffolds to facilitate future clinical translation.

It is interdisciplinary for osteochondral repair and regeneration involving biomaterials, cells, signaling factors, etc. Thus, integrative approaches should be considered to take advantage of each methodology. With continuous advances in anatomical, biological and physicochemical sciences at the osteochondral unit, it is expected that an appropriate disease model and the evaluation of the treatment and regeneration of the osteochondral unit can be established. Despite existing difficulties in the preclinical and clinical work, we expect further advancing TE strategies to gradually tackle these problems and eventually achieve the clinical translation of osteochondral tissues and scaffolds without life-long treatment or revision surgeries.

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Stem Cells Therapy for Cartilage Regeneration in Clinic: Challenges and Opportunities

17

Mina Shahnazari, Sara Malih, Reza Naeimi, Marzieh Savari, Niloofar Shokrollah, Parisa Samadi, and Mohsen Sheykhasan

Abstract

For both academics and clinicians, the repair and regeneration of articular cartilage have offered a challenging array of issues. Injuries to articular cartilage have a poor chance of healing since it is an avascular tissue. Small defects may eventually heal on their own without treatment, but the repair tissue is inferior to the body's own hyaline cartilage because it is made of fibrocartilage. Due to its regenerative capabilities, the idea of stem cell therapy has sparked intense research into its potential application for treating cartilage lesions, including OA. The purpose of this chapter is to present a perspective on stem cell-based therapy for cartilage repair and to highlight recent developments in advanced cell therapy, in particular, the use of embryonic stem cells, mesenchymal stem cells, and induce pluripotent stem cells for treating diseases associated with cartilage defects, particularly OA.

M. Shahnazari · R. Naeimi · M. Savari · N. Shokrollah

Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

S. Malih

Department of Radiology, University of Wisconsin-Madison, Madison, WI, USA

Department of Medical Biotechnology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

P. Samadi

Department of Operating Room, School of Nursing and Midwifery, ShahidBeheshti Hospital, Isfahan University of Medical Sciences, Isfahan, Iran

M. Sheykhasan (✉)

Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

Department of Mesenchymal Stem Cells, The Academic Center for Education, Culture and Research, Qom, Iran

Keywords

Stem cell · Regeneration · Cartilage · Osteoarthritis

17.1 Introduction

Articular cartilage is a crucial weight-bearing structure in joints (Simon and Jackson 2018). Synovial joints have a limited self-healing ability after cartilage damage due to the lack of blood vessels, nerves, and lymphatic vessels and the presence of dense extracellular matrix (ECM) (Armiento et al. 2019). If left untreated, damage to the articular cartilage can lead to osteoarthritis (OA), which is a progressive and irreversible form of arthritis and a leading cause of disability worldwide (Musumeci et al. 2015). OA is one of the most common musculoskeletal disorders and the most common rheumatic disease that leads to destruction of articular cartilage resulting in pain and stiffness, and in advanced stages may cause loss of joint movement (Lespasio et al. 2017). Although OA is associated with aging, other important risk factors may play a role in its manifestation, including gender, obesity, age, genetics, joint injury/trauma, overuse of joints, and underlying anatomical and orthopedic disorders. As well as other comorbidities such as diabetes, metabolic, and endocrine diseases (Musumeci et al. 2015).

In general, articular cartilage degradation affects nearly 60% of OA patients, making them candidates for arthroscopic surgery. However, few approaches are available to treat focal cartilage lesions (Palazzo et al. 2016). Although any joint in the body can be affected, patients mostly suffer from pain in the knees, buttocks, hands, ankles, and neck. The effects of orthosis, especially on the knee joints, are problematic for daily activities of patients, and treatment and improvement of OA-related musculoskeletal disorders will become more important as life expectancy increases in the next few decades (Collins et al. 2019).

Pathologically, the main cause of OA is cartilage destruction, imbalance between catabolism and anabolism, increased activity of signaling catabolic pathways, and ECM degrading enzymes, leading to hypertrophic differentiation of cartilage, as well as vascular and matrix destruction (Li et al. 2017). From a molecular point of view, the aging process of cartilage cells (chondrocytes) shows a decrease in response to growth factors with more expression of inflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin 1 (IL-1), which requires increased synthesis of matrix metalloproteinases (MMPs) and leads to ECM destruction. In addition, OA can cause changes in cartilage signaling activity, leading to a hypertrophic phenotype by increasing the differentiation process (Boehme and Rolauffs 2018).

Current therapeutic approaches for early and moderately early OA include pharmaceutical and nonpharmacological treatments as well as surgical approaches, which all failed to repair cartilage lesions effectively and have shown poor regeneration (Medvedeva et al. 2018). Cartilage repair strategies currently used in the clinic cannot completely regenerate hyaline, and lack of effective repair can lead to

extensive joint destruction associated with OA (Roseti et al. 2019). Hence, new stem cell-based therapies have developed with the potential to treat patients with cartilage damage. In numerous preclinical and clinical studies, cell-based therapies have shown greater potential of re-differentiation into chondrocytes and form hyaline cartilage for better control of symptoms (Bianchi et al. 2017). Moreover, compared to autologous cartilage cells, stem cells have a wider source and a stronger ability to expand *in vitro*, which makes tissue-engineered cartilage containing stem cells more advantageous than those containing autologous cells (Jiang et al. 2020). Stem cell-based tissue engineering strategies include implantation of exogenous stem cells and housing of endogenous stem cells to achieve *in situ* cartilage regeneration. The basis of exogenous stem cell culture is to find suitable types of stem cells, with the ability to differentiate into chondrocytes (De Bari and Roelofs 2018). In articular cartilage engineering, mesenchymal stem cells (MSCs) derived from different tissues are commonly used in recent clinical and preclinical studies (Li et al. 2018). Moreover, embryonic stem cells (ESCs) have the potential to transform into different cells, but they are only in the preclinical phase (Ma et al. 2018).

Induced pluripotent cells (iPSCs) can theoretically be obtained by reprogramming each type of differentiated cell. However, in stem cell transplantation there are risks of tumorigenesis, immune rejection, disease transmission, and functional heterogeneity of cells from different individuals (Yamanaka 2020). The ongoing development of tissue engineering strategies also seeks to combine stem cells with different scaffolds and cartilage signals to produce a functional tissue that can be used to repair cartilage damage. However, there are new challenges to such treatments that need to be addressed as our knowledge advances (Yamagata et al. 2018).

17.2 Stem Cells for Cartilage Regeneration

Over the last decades, cell/stem cell-based therapeutics have emerged as an effective strategy in different fields of regenerative medicine. In this regard, an ideal cell therapy should be available and accessible, viable, nonimmunogenic, nontumorigenic, and being responsive to different bioactive factors. Therefore, in cell therapy strategies for cartilage repair, stem cells of different origins rather than chondrocytes have been investigated (Fig. 17.1) (Zhang and Lai 2020).

In recent years, stem cell-based therapeutics have shown promise in cartilage regeneration by avoiding the disadvantages associated with traditional chondrocyte therapies. Different types of stem cells commonly used for cartilage repair are ESCs, iPSCs, and MSCs, which have many advantages for clinical applications due to their chondrogenic potential (Ma et al. 2018). These stem cells have the potential to differentiate into bone and cartilage, indicating their regenerative capability for cartilage *in vitro* and *in vivo* (Wang et al. 2020; Khajeh et al. 2021; Lee et al. 2021). Regarding ESCs, they grow indefinitely and thus provide an unlimited source of cells with the ability of differentiating into chondrogenic cells. Moreover, the regenerative potential of iPSCs for chondrocyte differentiation is also gaining great attention in the course of recent years (Griffith et al. 2021). In this purpose, there are

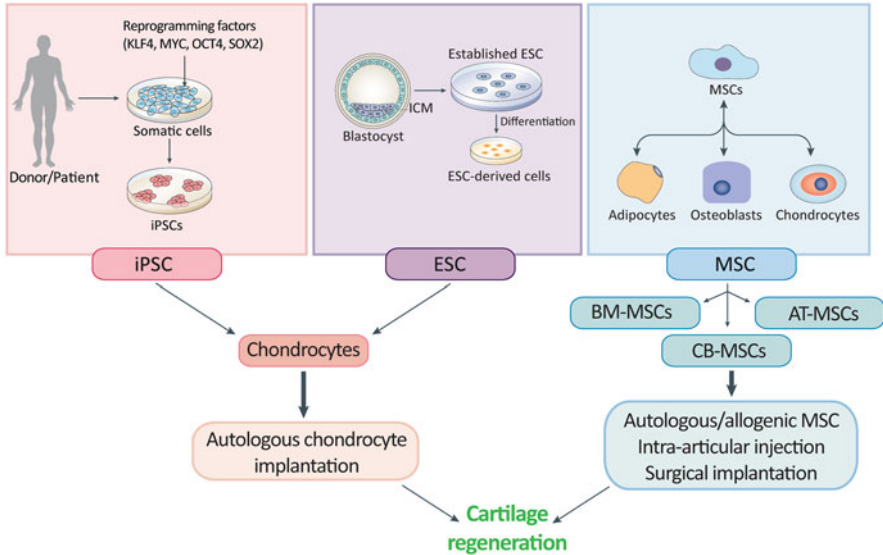


Fig. 17.1 Different sources of stem cells for cartilage regeneration

robust protocols for induction of chondrogenesis including coculture with primary chondrocytes, embryoid bodies formation, intermediate MSC cellular differentiation (Ferreira et al. 2021; Im 2022).

Although both adult ESCs and iPSCs are potential stem cell sources for cartilage regeneration and repair, however, their lower efficiency of differentiation causes an important hurdle for the clinical implementation (Urlić and Ivković 2021). In this regard, MSCs from adult somatic cell types have several superior benefits over other adult stem cells such as less ethical and safety concerns regarding their clinical applications, which make them as the dominant stem cell sources in clinical trials for cartilage regeneration (Urlić and Ivković 2021; Lee and Wang 2017).

MSCs from bone marrow (BM-MSCs) were the first type of stem cells considered for effective regenerative therapies (Samadi et al. 2021). Adult MSCs have attracted significant interest in regenerative medicine since they have unique properties including their presence in many organs and tissues, easy to obtain with no ethical considerations, potent immunosuppressive effects, and safety without any possibility of malignancy (Berebichez-Fridman and Montero-Olvera 2018). With regard to different MSCs' populations, one of the frequently applied MSCs in the repair and regeneration of articular cartilage defects is BM-MSCs, which have been used in a wide range of small and large animal models. Although BM-MSCs have demonstrated positive effect on cartilage repair and regeneration, their highly invasive donation procedure as well as the limited yield of stem cells during this process encourages the search for alternative tissue sources of MSCs (Jia et al. 2018; Zhang et al. 2020). On the other hand, substantial amounts of adipose tissue-derived mesenchymal stem cells (AT-MSCs), for example, can be relatively easy to isolate,

and their intrinsic properties not being affected by donor-related aspects, such as gender and age (Frese et al. 2016). However, compared to BM-MSCs, AT-MSCs represented a significantly lower potential for osteogenic and chondrogenic differentiation both in vitro and in vivo (Teunissen et al. 2021; Neybecker et al. 2020).

17.3 Stem Cell Delivery Strategies in Cartilage Regeneration

In terms of delivery strategies, many studies used an intra-articular injection of scaffold-free stem cells, with the major advantage of simple administration, but it may only be beneficial in early stages of cartilage injury. Furthermore, intra-articular injection may cause cell dispersion and an insufficient number of stem cells required for repair (Zhang et al. 2019). One way to solve this issue is by employing a local adherent technique for transplantation of MSCs to the cartilage defect. However, by using these methods, the transplanted MSCs lack an ECM, which is crucial for cells to exploit their important functions (cell proliferation and differentiation) (Gomez et al. 2020). To address this, novel 3D environment and tissue-engineered constructs containing native ECM, synthesized by MSCs have been developed in recent years. These 3D scaffolds with tunable pore size, excellent biocompatibility, and biodegradability, then can be implemented through a minimally invasive surgery (Liu et al. 2018). In addition, 3D scaffolds should provide other advantages such as adequate strength and rigidity of mechanical properties, improved transportation of gases and nutrients, and adhesion to the cartilage defect (Cipollaro et al. 2019).

In order to promote cartilage repair and regeneration, stem cells of different origins have to be combined with a wide variety of natural and synthetic biomaterials. Of these carriers, the most frequently explored are hydrogels, which are water-swollen, and cross-linked polymeric networks produced by the simple reaction of one or more monomers (Xu et al. 2019). Hydrogels facilitate diffusion of stem cells and required nutrients which could significantly increase ECM and chondrocyte synthesis (Deng et al. 2020). Moreover, other scaffolds such as Poly (N-isopropylacrylamide) have represented to produce similar structures to the natural cartilage with enhanced chondrogenesis of MSCs (Wang et al. 2018a).

In order to make various human stem cells appropriate for human clinical trials, we need a more in-depth knowledge about their behavior inside the body on larger immunocompetent animals. In this regard, in several studies they have reported the use of human MSC transplantation in smaller nonimmunosuppressed animals without any graft rejection. Regarding larger animal models such as immunocompetent dog, transplantation of MSCs have also shown similar results and even indicated immunosuppressive capacities related to these MSCs (Lo Monaco et al. 2018; Wang et al. 2018b).

In this case, Dayan et al. have provided the evidence for safe and efficient MSCs transplantation in ovine immunocompetent animal models, signifying the preclinical large animal model for testing human stem cells (Dayan et al. 2016). However, the triggering an immunogenic response following human stem cell transplantation into the animal model, can be generally addressed via implementing immunosuppressive

drugs at the time of MSC transplantation. Otherwise, the use of autologous MSC transplantation can be considered. However, there may be several drawbacks for using autologous stem cells such as difficulties of growing MSCs in vitro and time-consuming procedure to obtain an adequate number of MSCs (García-Bernal et al. 2021). In relation to cartilage regeneration, these difficulties left the field open for advancement of more potent and novel stem cell-based therapies to repair the damaged cartilage tissue. However, due to the fact that the number of endogenous MSCs available for chondrocyte differentiation are limited, external MSC transplantation could be an effective strategy to improve the joint cartilage repair and regeneration (Gupta et al. 2012). Therefore, beyond any doubt, for translational research in stem cell therapy of cartilage repair, studies in preclinical animal models which are immunocompetent should gain more attention.

17.4 Mechanism of Stem Cells' Function in Cartilage Regeneration

Stem cells, which have been studied from a variety of sources with varying differentiation capabilities, are among the most promising and attractive candidates in regenerative medicine. Numerous studies have shown that these cells can be effective in regenerate damaged tissue, including cartilage regeneration through various mechanisms including: homing, angiogenesis, differentiation, and response to inflammation (Kangari et al. 2020a). It was initially thought that stem cells could regenerate cartilage through direct differentiation into chondrocytes. Further studies have shown that in addition to differentiation, stem cell secretome can also be effective in tissue regeneration (Fig. 17.2). These secretomes can affect surrounding cells and tissues and have paracrine effects. In addition, stem cells have conditioned media (CM), extracellular vesicles (EVs), and other valuable derivatives, all of which can be effective in tissue regeneration (Jiang et al. 2021; Fayazi et al. 2021; Khoei et al. 2020). Therefore, the various mechanisms through which these cells can be effective in regenerating cartilage will be discussed.

17.5 The Effect of Stem Cells on Cartilage Regeneration by Differentiating and Affecting Biological Processes

Stem cells can differentiate into matrix-producing chondrocytes, while a number of these cells can maintain their pluripotency and ensure the differentiation process (Wang et al. 2019; Zhou et al. 2019). Numerous studies show that intra-articular injection of mesenchymal stem cells differentiates these cells into cells with the chondrocyte phenotype (Caldwell and Wang 2015). For example, in the study of Abir et al. (Mokbel et al. 2011), which was conducted in order to investigate the therapeutic effects based on the homing ability of MSCs in animal models suffering from osteoarthritis, it was determined that these cells can reach the damaged joints after intra-articular (IA) injection and cause cartilage regeneration and improve

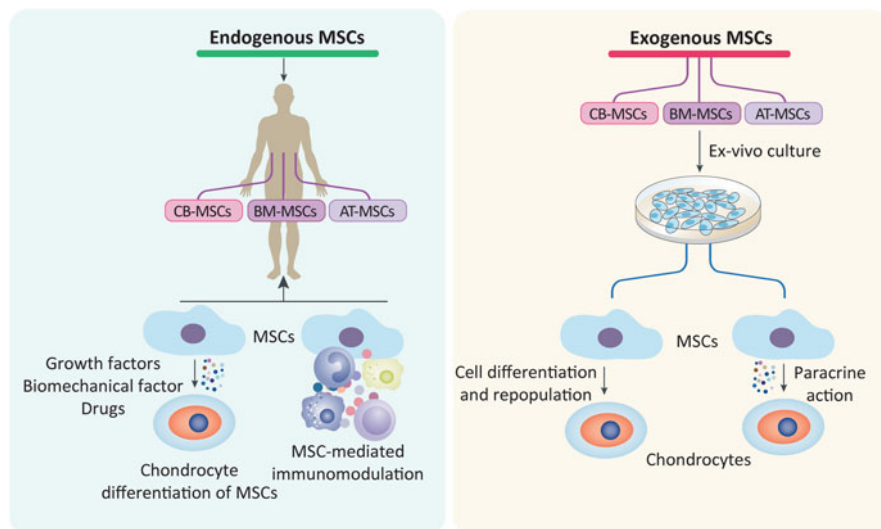


Fig. 17.2 Mechanisms of exogenous and endogenous MSCs for cartilage regeneration

osteoarthritis. Further studies showed that in many cases, cell therapy for direct differentiation did not have very promising results, and implanted MSCs were removed from the target tissue after some time. For example, Tommy et al. follow-up of implanted allogeneic MSCs reported that after 12 months of treatment, no allogeneic MSC DNA could be detected, indicating that MSCs eventually die and are removed from tissue (de Windt et al. 2017). According to this research, stem cells' ability for tissue repair and regeneration is likely mediated by active substances they secrete rather than by directly differentiating into target cells. Recent studies suggest that stem cells may be more useful in cartilage regeneration through their paracrine effects on biological processes (Jiang et al. 2021). In other words, although stem cell differentiation can be effective in repairing target tissue, the active components secreted by these cells have more tangible effects.

Research has shown that the CM of stem cells as well as the types of signaling molecules such as cytokines that are secreted from them can play a role in both protecting and repairing damaged tissues (Caplan and Dennis 2006; Pawitan 2014). It has also been shown that stem cells are involved in regenerating damaged tissue by regulating the immune microenvironment (Li et al. 2019). Numerous in vitro studies have shown that MSCs can have significant effects on chondrocytes through the secretion of signaling molecules and improve the healing process (Acharya et al. 2012; Wu et al. 2011; Zhang et al. 2018). Of course, the diversity of data and studies and results indicate an almost complex process in cartilage regeneration by stem cells. Stem cell secretions can have a variety of effects at different stages of repair, and as mentioned, the type of stem cell is very important. ECM, EVs, and CM should be more accurately evaluated for stem cells used in studies to more accurately identify repair mechanisms.

17.6 Regeneration Mechanisms of Mesenchymal Stem Cells in Damaged Cartilage

As mentioned, MSCs have been evaluated and researched more than other stem cells in in vitro and in vivo studies in regenerative medicine and cartilage regeneration. Therefore, in this section, we will take a closer look at the mechanisms associated with these cells.

MSCs can contribute to the improvement of cartilage damage in two ways: first, through the secretion of effective factors in preventing cartilage destruction and secretomes that can have cartilaginous effects (Table 17.1). The second is through the differentiating ability of these cells to reproduce chondrocytes (Akgun et al. 2015; Chen et al. 2013a).

A few cytokines play a key role in cartilage disease. These cytokines include pro-inflammatory cytokines including TNF-, IL-6, IL-1, and IL-17 (Liu et al. 2010). PGE2, a crucial component in MSCs' capacity to control immune responses, can inhibit T cells from proliferating and from secreting these cytokines, which delays the progression of the inflammatory process (Chen et al. 2010; Jorgensen and Noël 2012).

Osteoarthritis (OA) is one of the most important inflammatory diseases of the joints and is very common. High levels of metalloproteinase-2 (MMP-2), MMP-9, and MMP-13 have been observed in this disease (Jackson et al. 2014). These enzymes damage the ECM, so preventing them from working will protect tissue

Table 17.1 Effects of MSCs' secretomes on cartilage regeneration

MSCs' secretome	Mechanism	Reference
PGE2	Prevent the secretion of TNF- α , IL-6, IL-1 β , and IL-17	Chen et al. (2010); Jorgensen and Noël (2012)
TIMP-2	Prevent MMP-2	Jackson et al. (2014)
TIMP-1	Prevent MMP-9	Jackson et al. (2014)
HGF	Prevent fibrosis and apoptosis of chondrocytes	Lemos Dario and Duffield Jeremy (2018)
EVs	IL-10 secretion of B cells	Cosenza et al. (2017)
Exosome	Reduce the expression of inflammatory genes	Tao et al. (2017); Wang et al. (2017)
Exosome	Stimulate the expression of type II collagen and aggrecan, inhibiting ADAMTS5	Zhu et al. (2018)
TGF- β	Synthesis of collagen type II and proteoglycans	de Kroon et al. (2016); Lee et al. (2016)
TSP2	Differentiation of cartilage	Jeong et al. (2013, 2015)
VEGF	Chondrogenic differentiation and cartilage matrix formation	Freitag et al. (2016)
EGF	Chondrogenic differentiation and cartilage matrix formation	Freitag et al. (2016)

damage. MSCs secrete inhibitor of metalloproteinases 2 (TIMP-2) and TIMP-1, which prevent MMP-2 and MMP-9 respectively, thus protecting the cartilage ECM (Jackson et al. 2014). ECM synthesis also depends on the proliferation and number of chondrocytes. MSCs are able to protect chondrocytes against fibrosis and apoptosis by secreting hepatocyte growth factor (HGF), thereby increasing chondrocytes and increasing the proliferation of these cells (Lemos Dario and Duffield Jeremy 2018). Studies have shown that increased IL-10 secretion of B cells reduces inflammation and pathological changes in cartilage-related diseases. Research has shown that EVs derived from MSCs can increase IL-10 secretion (Cosenza et al. 2017). Exosomes, which are nanometer EVs, have recently been considered in many studies related to MSCs. For example, it has been shown that exosomes derived from MSCs can reduce the expression of inflammatory genes such as IL-1 β , thereby disrupting the inflammatory process in OA (Tao et al. 2017; Wang et al. 2017). Also, studies have shown that exosomes derived from BM-MSCs stimulate the expression of type II collagen and aggrecan, while inhibiting markers such as ADAMTS5, thereby playing a role in reducing the inflammatory process in OA (Zhu et al. 2018). Signaling molecules such as transforming growth factor beta (TGF- β) can be involved in the differentiation of MSCs and chondrogenic reproduction. In this differentiation pathway, TGF- β 2, TGF- β 1, and TGF- β 3 are of particular importance because they are involved in the synthesis of important proteins such as collagen type II and proteoglycans (Lee and Wang 2017; Puetzer et al. 2010). The downstream molecules of this signaling pathway are Smad2/3, which, if phosphorylated by TGF- β , can go to the nucleus, affect transcription factors such as SOX9 and collagen type II (COL II), and ultimately direct differentiation (de Kroon et al. 2016; Lee et al. 2016). Other signaling pathways that are involved in this process of differentiation and chondrogenesis are the Wnt/ β -catenin and MAP kinases (Kang et al. 2012; Luo et al. 2013). In connection with these signaling pathways, the differentiation of chondrocytes from progenitor cells is enhanced by protein kinase C alpha (PKC α), extracellular signal-regulated kinase (ERK), p38/MAPK, and notch signaling pathways. This important signaling process is promoted by one secretion of MSCs called Thrombospondin (TSP2). This molecule is an important regulatory factor in the differentiation of cartilage and can also play a role in the differentiation of bone cells (Jeong et al. 2013, 2015). Vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) are other important molecules that can be involved in the differentiation process of MSCs as well as in chondrogenic differentiation. In addition, it has been shown that these important factors also play a role in cartilage matrix formation (Freitag et al. 2016).

As is well known, the general mechanisms described for cartilage regeneration for stem cells also apply to MSCs, and this important type of stem cell can often play a role in cartilage regeneration through paracrine effects. On the other hand, accurate understanding of the signaling mechanisms of MSCs in the process of tissue regeneration is a goal that is still being pursued by researchers.

17.7 Embryonic and Induced Pluripotent Stem Cells in Cartilage Regeneration

At first, we need to define embryonic stem cells (ESCs) and induced pluripotent stem (iPS) cells. ESCs are obtained from the inner cell mass of the human blastocyst, an early stage preimplantation embryo lasting 4–7 days postfertilization (Toh et al. 2011). ESCs are known with unlimited capacity for self-renewal as well as the potential to differentiate into all other somatic cell types (Klimanskaya et al. 2020). iPS cells, are a new type of pluripotent stem cell obtained by genetically reprogramming of adult somatic cells to an embryonic stem (ES) cell-like state to develop an unlimited source of all type of human cells but lack the ethical concerns associated with the use of ESCs (Toh et al. 2009). Here, we discuss the mechanisms of action of stem cell-based therapies for cartilage regeneration.

17.7.1 Embryonic Stem Cells (ESCs)

Successful application of hESCs for cartilage regeneration will depend on the ability to develop expandable and homogenous population of chondrogenic cells that can generate cartilaginous tissue in a well-defined system. So far, several strategies toward improving the potential of ESCs in cartilage repair have been applied that include growth factor, coculturing, and the addition of conditioned medium, small molecule, genetic manipulation, biomaterial, and biophysical stimulations (Toh et al. 2011; Jonidi Shariatzadeh et al. 2018). These include culturing in a defined medium to which chondro-inductive cytokines, growth factors, and chemicals have been added, coculturing, and the addition of conditioned medium (Nakayama et al. 2021).

17.7.1.1 Growth Factors

One of the strategies for optimizing the local milieu for controlling the chondrogenic differentiation of ESCs is the application of exogenous growth factors and cytokines. Such factors include TGF- β (Cancedda et al. 1995), bone morphogenetic protein (BMP), fibroblast growth factor 18 (FGF18) (Teunissen et al. 2021; Neybecker et al. 2020), insulin-like growth factor 1 (IGF1) (Koay et al. 2007), and stromal cell-derived factor 1 (SDF1/CXCL12) (Liu et al. 2018; Cipollaro et al. 2019), PDGF-bb (Nakayama et al. 2003), and SHH (Hoben et al. 2009). Using growth factors and cytokines, it is important to consider growth factors combination, their concentration and time of administration, as well as developmental stage and cell populations induced, together with the appropriate culture system. Previous studies in mESCs demonstrated that growth factors applied at different stages of EB differentiation influenced the range of chondrogenesis, and ESCs may have to be differentiated to precursor cells to induce the chondrogenic fate (Kramer et al. 2000). Furthermore, multiple studies revealed that BMPs and TGF- β 1/3 could induce chondrogenic differentiation of hESCs via EB formation and differentiation (Koay et al. 2007; Nakagawa et al. 2009; Toh et al. 2009). Some other studies evaluated the chondrogenesis potential of hESCs in micromass cultures without the EB formation

and observed that TGF- β 1 induces chondrogenic differentiation only in the presence of BMP-2 (Yang et al. 2009; Gong et al. 2010). However, early addition of the TGF- β 1 to the undifferentiated hESCs prohibits chondrogenesis (Yang et al. 2009) which could be due to the TGF- β /activin/nodal-signaling role in conserving the pluripotency and undifferentiated state of hESCs (James et al. 2005). Unlike the TGF- β 1, BMP2 induced direct chondrogenic differentiation of hESCs in high-density micromass cultures that proposes a different mechanism of chondrogenesis induction (Yang et al. 2009; Gong et al. 2010; Roark and Greer 1994). On the other hand, several growth factors and cytokines instigate numerous intracellular signaling mechanisms, which may result in pleiotropic effects on stem cell differentiation that could become more entangled with the presence of serum with undefined growth and differentiation factors and may change the cell fate to various lineages other than the chondrogenic lineage (Zur Nieden et al. 2005; Toh et al. 2007). Yamashita et al. demonstrated that under minimal serum conditions and in the presence of TGF- β 1 and BMP-2, induction of chondrogenesis and formation of cartilage were improved compared to high serum conditions and TGF- β 1 and BMP-2 (Yamashita et al. 2009). Gertow et al. showed that Activin/Nodal, FGF, and WNT play a role in differentiation of mesoderm from hESCs (Gertow et al. 2013). In a recent study by Wang et al., it was observed that BMP2 induces chondrogenic differentiation from hESCs more than BMP4 does, and the effect is more durable (Wang et al. 2019). There are few studies on human embryonic stem cell (hESC) differentiation by growth factors into chondrocyte progenitors, which are mostly limited to preclinical (Table 17.1).

17.7.1.2 Coculture, Conditioned Medium, and Morphogenetic Factors

In this context, using direct coculture with Limb bud progenitor cells of a developing embryo, Sui et al. found that cocultured mESCs could form Alcian blue-positive cartilage nodules (Sui et al. 2003). In another study, Bigdeli et al. demonstrated that coculture of hESCs with irradiated neonatal or adult articular chondrocytes improved cartilage matrix production in vitro (Bigdeli et al. 2009).

Due to the risk of contamination from the coculture cell type that may pose an impact on the yield and purity of the differentiated cells, the conditioned medium approach seems more appropriate. Using hESCs from patient-derived human chondrocytes (Vats et al. 2006) or primary explanted bovine chondrocytes (Hwang et al. 2008a), along with morphogenetic and differentiation factors released from the mature chondrocytes, chondrogenic cells with the ability to grow and form cartilage tissue were produced (Hwang et al. 2008a). Another strategy to improve chondrogenic differentiation of ESCs is mesodermal preinduction. Hwang et al. demonstrated that pretreatment of mESCs with the human hepatocarcinoma cell line (HepG2)-CM resulted in increased mesoderm formation and enhanced chondrogenic differentiation (Hwang et al. 2008b). There are numerous studies that have used coculture and conditioned medium as a strategy to induce chondrogenic differentiation in ESCs (Hwang et al. 2008a; Karlsson et al. 2009; Hill et al. 2010). In a study conducted on the lavage fluid of the knee joints of 47 patients, H. Schmal et al. investigated the relationship between the expression of bone morphogenetic proteins 2 and 7 in the repair of limited cartilage lesions and

clinical outcome. They found that BMP2 was the only intra-articular growth factor that correlated with clinical outcome, suggesting that it may be crucial in surgically induced cartilage regeneration (Schmal et al. 2010). In vitro studies have confirmed osteoinductive capacity of recombinant human BMP (rhBMP) in cartilage regeneration and it has been evaluated in clinical trials. So, it was observed that rhBMP-2 plays a more important function in subchondral bone healing than rhBMP-4 when it comes to the repair of hyaline cartilage in the rabbit osteochondral defect model (López-Morales et al. 2010). Also, Taniyama et al. evaluated the effect of transplantation of porous hydroxyapatite collagen (HAp/Col) impregnated with rhBMP-2, and found it to be effective in cartilage repair (Taniyama et al. 2015). There are still a lot of challenges that need to be considered before translation to clinical application, such as risks of pathogen transmission, poor definition of components related to inducing differentiation, and lack of standardization.

17.7.1.3 Small Molecules

Small molecule drugs are essential in stem cell chondrogenesis. The impact of the concomitant application of small molecules including ascorbic acid, dexamethasone, and sodium pyruvate with growth factors stimulators including TGF- β and BMPs on chondrogenic differentiation of chondrocytes (Liu et al. 2007), adult MSCs (Yoo et al. 1998; Toh et al. 2005), and ESCs (Kramer et al. 2000; Toh et al. 2009, 2007) have been widely investigated.

There is still much to know about the effects of small molecule drugs on chondrogenic differentiation of ESCs. Kawaguchi et al. demonstrated the booster effect of retinoic acid on neural crest differentiation of ESCs (Kawaguchi et al. 2005). In an exciting differentiation protocol, Lauren Foltz et al. demonstrated self-organizing craniofacial cartilage organoids can be generated from human embryonic stem cells (hESCs) and iPSCs via a neural crest stem cell (NCSC) (Foltz et al. 2021). Furthermore, Zhang et al. observed that reorganization of actin filaments by addition of cytochalasin improves chondrogenic differentiation of mouse embryoid body (EB)-derived cells and mESCs (Zhang et al. 2006).

17.7.1.4 Genetic Manipulation

Another approach to improve chondrogenesis is the application of stable mESC lines generated by genetic manipulation. Sox9 is a master gene in the regulation of chondrogenesis that controls the expression of collagen II, IX, and XI, as well as aggrecan (Bell et al. 1997; Lefebvre et al. 1997; Bridgewater et al. 1998; Sekiya et al. 2000). It was observed that in mESC cell lines overexpressing Sox9 gene, chondrogenesis with features of adult collagen isoform, Col IIB, was improved and observable by day 3 (Kim et al. 2005), but the same effect was not seen in normal wild type 5-day (5'd') EBs until day 14 in serum-supplemented medium (Tanaka et al. 2004). Hargus et al. demonstrated that chondrogenic differentiation of mESCs would be impaired in the absence of Sox9 in vitro (Hargus et al. 2008). Cheng and colleagues showed that chondrogenic cells derived from hESCs improved cartilage repair via high expression of SOX9 (Cheng et al. 2014a). In addition to Sox9 as a major activator of chondrogenic differentiation, a combination

of Sox5, Sox6, and Sox9 signaling molecules are required to instigate hyaline cartilage in mice ESCs that could be a prosperous approach in cartilage repair (Matsumura et al. 2004). In a study done by Cheng et al., they observed that the recombinant ECM molecules, fibronectin, and fibronectin fragment (FN III), as well as the fibrillin-1 fragment PF8, can be used for hESC chondrogenesis instead of the application of plasma FN and gelatin (Cheng et al. 2017). Until now, most of the studies in the field of genetic manipulation have been done on MSCs, but well-defined differentiation protocols with good in vivo results could soon provide a strong foundation for building clinical trials in that area.

17.7.1.5 Biomaterial-Assisted Chondrogenic Differentiation of ESCs and Cartilage Tissue Engineering

There are both natural biomaterials and synthetic biomaterials available for utilizing in chondrogenic differentiation, including alginate, agarose, gellan gum, cellulose, hyaluronic acid (HA), collagen, gelatin, fibrin, and decellularized tissue matrices as natural biomaterials and poly(ethylene glycol) (PEG), poly(lactic-co-glycolic acid) (PLGA), and poly (L-lactic acid) (PLLA) as synthetic biomaterials (Toh et al. 2011; Jelodari et al. 2022). It is observed that mouse EBs encapsulated in PEG-based hydrogels will improve chondrogenesis (Hwang et al. 2006a) and cartilage matrix synthesis (Hwang et al. 2006b). In the hyaluronic acid hydrogel system, hESCs' chondrogenic cells differentiated to hyaline-like cartilaginous tissue (Toh et al. 2010). In a study by Jukes et al., it was observed that intact EBs or EB-derived cells from mESCs could differentiate to chondrocytes on poly(ethylene oxide terephthalate)-poly(butylene terephthalate) (PEOT/PBT) scaffolds (Jukes et al. 2008). Levenberg et al. demonstrated that utilizing PLGA/PLLA polymer scaffolds improves differentiation of hESCs when seeded in combination with matrigel on the scaffold (Jukes et al. 2008). In a similar way, Vats and colleagues showed that hESC-derived chondrogenic cells seeded on the PLLA scaffold induce chondrogenesis with s-GAG and collagen II (Vats et al. 2006). In another study, using alginate and PLGA scaffolds, human EBs were differentiated to heterogeneous cartilaginous tissue (Bai et al. 2010). Altogether, these studies reveal that there is a great need in developing novel approaches to improve differentiation protocols to obtain homogenous cartilage tissue.

17.7.2 Induced Pluripotent Stem Cells

In contrast to hESCs, human-induced pluripotent stem cells (hiPSCs) are reprogrammed somatic cells to a pluripotent state using the overexpression of a key set of reprogramming factors, referred to as Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc also called OSKM) (Kim et al. 2022). HiPSCs have presented undeniable potential in the field of cartilage tissue engineering since, unlike hESCs, the application of iPSCs can be personalized for each patient and eliminate ethical concerns (Wert and Mummery 2003; Lee et al. 2009). HiPSCs have similar features to embryonic stem cells, including infinite self-renewal and proliferation properties

as well as the ability to differentiate into all the three germ layers: mesoderm, endoderm, and ectoderm (Bruschi et al. 2022; Vonk et al. 2015). Part of the issues related to hESCs can be avoided by using iPSCs. iPSCs are a good source of patient-specific cells for application in autologous tissue regeneration. Various in vitro studies have revealed the potential of using iPSCs derived from various cell types in cartilage engineering (Qu et al. 2013; Medvedev et al. 2010; Nam et al. 2017; Li et al. 2016).

Rodríguez Ruiz et al. studied the generation of neo-cartilage from hiPSC with features similar to chondrocytes from human primary articular chondrocytes (hPACs) or human bone marrow-derived mesenchymal stromal cells (hBMSCs) and found that after 21 days of chondrogenesis from hiPSCs via chondroprogenitor cells, considerable similarity to primary chondrocytes can be observed (Rodríguez Ruiz et al. 2021). Transient receptor potential vanilloid 4 (TRPV4) is overexpressed in cartilaginous tissues. Willard et al. studied the role of TRPV4 in chondrogenesis of miPSCs and observed that the expression level of TRPV4 and the chondrogenic gene markers Sox9, Acan, and Col2a1 were increased (Willard et al. 2021). According to these results, they suggested TRPV4 as a regulator of iPSCs chondrogenesis. Wu et al. analyzed single-cell transcriptomic of hiPSCs chondrogenesis and demonstrated that inhibition of WNTs and MITF enhanced the yield and homogeneity of hiPSC chondrogenesis (Wu et al. 2021).

Numerous studies have used patient-derived hiPSCs to introduce novel therapeutic approaches (Nam et al. 2018; Kamaraj et al. 2021), including in vitro (Koci et al. 2017; Sirenko et al. 2013; Hofrichter et al. 2017; Kondo et al. 2017) and preclinical (Ozay et al. 2019; Doi et al. 2020; Sundberg et al. 2013) studies. So far, various methods have been proposed for differentiating hiPSCs toward articular chondrocytes (Castro-Viñuelas et al. 2018; Murphy et al. 2018), including (1) the coculture with chondrocytes (Vats et al. 2006; Driessen et al. 2017), (2) differentiation through the generation of MSC-like cells from hiPSCs (Nejadnik et al. 2015; Hontani et al. 2019), (3) application of specific proteins and growth factors, (4) genetic manipulation, and (5) biomaterials.

For the coculture of hiPSCs, Cheng et al. used differentiated mature chondrocytes (Cheng et al. 2014a); although, the success of this method is closely related to the quality of primary cells and the other limitation is that the chondrocytes have a tendency to phenotype loss in culture (Lietman 2016). The generation of MSCs from hiPSCs is the second technique. In this approach, hiPSCs are cultured in MSC culture medium to transform to MSC-like cells and then differentiate into chondrocytes. More extended culture period and fibrocartilage and hypertrophic cartilage formation are major limitations of this technique (Nejadnik et al. 2015; Cheng et al. 2014b; Zhu et al. 2016).

Using growth factors to induce chondrogenesis is another approach. Lee et al. added WNT3A, Activin A, FGF-2, and BMP-4 on different days to differentiate hiPSCs to embryoid bodies and then mesoderm. In this study, for chondrogenesis induction, follistatin, NT4, and GDF5 were used, and chondrocytes formation was observed after 14 days (Lee et al. 2015). In another study, Adkar et al. added Activin A, CHIR99021, and the FGF-2 succeeded by BMP inhibitor (dorsomorphin)

and TGF- β inhibitor (SB505124) to induce mesodermal lineage differentiation and sclerotome formation and then applied BMP-4 to hiPSCs for prechondrogenesis followed by addition of TGF- β 3 for mature chondrogenesis (Adkar et al. 2019). Lee et al., demonstrated that chondrogenic induction of mesodermal cell-derived chondrocytes (MC-Chs) and neural crest cell-derived chondrocytes (NCC-Chs) with TGFB1 and some selected growth factors improved differentiation of hyaline cartilage chondrocytes from MCs and NCCs (Lee et al. 2021).

Kawata et al. observed that application of two small molecules, CHIR99021 with TTNPB (a substitute of Activin A), could differentiate hiPSCs to chondrocytes that expressed SOX9, SOX5, Collagen II α 1, and Collagen XI α 2 chondrogenic markers (Kawata et al. 2019). In a recent study, Dicks and colleagues used chondroprogenitor subpopulations expressing CD146+/CD166+/PDGFR β +/CD45– surface markers to obtain more homogenous and efficient hiPSC chondrogenic differentiation (Dicks et al. 2020). Genetic manipulation approaches could also be useful to improve chondrogenic properties or induce chondroprotective features. Seidl et al. applied the CRISPR-Cas9 editing tool to reduce MMP13 protein levels and enzymatic activity and improved the accumulation of type II collagen (Seidl et al. 2019). In another study, van Hoolwerff et al. revealed that missense mutation FN1 resulted in decreased binding capacity to collagen type II and subsequently reduced chondrogenic potential (Hoolwerff et al. 2021). Pretemer et al. developed a system of hypertrophic chondrocyte differentiation from iPSCs and evaluated the effect of heterozygous MATN3 and COL10A1 mutations on phenotype to model chondrodysplasias (Pretemer et al. 2021).

Several recent studies have applied biomaterials such as agarose (Diekman et al. 2012), alginate, cellulose (Nguyen et al. 2017), and fibrin (Goetzke et al. 2019) to enhance chondrogenic differentiation of hiPSCs. Using nanofibrillated cellulose/alginate bioink for 3D printing, Nguyen et al. improved hiPSCs differentiation toward chondrogenic features (Nguyen et al. 2017). Lee et al. applied a biomimetic hydrogel consisting of chondroitin sulfate and polyethylene glycol to generate cartilage tissue without teratoma formation (Lee et al. 2015). In another study done by Ko and colleagues, they utilized alginate gels with iPSCs-derived chondrocytes to improve deposition of proteoglycans and collagen II in a rat model of the osteochondral defect (Ko et al. 2014). Eventually, these studies represent hiPSCs-based tissue engineering therapeutics as prosperous approaches in cartilage repair and OA therapy in the future.

17.8 Mesenchymal Stem Cells in Cartilage Regeneration

As mentioned, MSCs are highly proliferative intracellular cells that, in addition to regenerative properties, can differentiate *in vitro* into lineage and mesoderm-derived cells (Caplan 2017). In other words, by defining MSC phenotypes *in vitro*, these cells could differentiate into osteoblasts, chondrocytes, adipocytes, and myocytes (Caplan 2007). Due to continuous efforts, a bright and promising horizon in cartilage regeneration using mesenchymal stem cells can be seen in basic research and clinical

trials. Recent studies on the origin and function of MSCs have revealed new dimensions that define the concept of mesenchymal stem cells as widely distributed cells derived from a subset of perivascular cells called the “pericytes” (Chen et al. 2015; Crisan et al. 2008), which is generally defined as “a group of contractile cells adjacent to the endothelium in the vascular basement membrane” (Crisan et al. 2008; de Souza et al. 2016). Thus, the perivascular origin of these cells is a good indication of the easy separation of these cells from almost any vascular tissue (Meirelles et al. 2006). Under normal circumstances, pericytes are at rest and have the least contribution to tissue homeostasis. Conversely, in the event of damage, leading to a change in the local environment, pericytes are forced to show the opposite side of the coin (It is activating their MSC profile) (Chen et al. 2013b; Soliman et al. 2021).

“Activated” pericytes, commonly referred to as MSCs, assist the tissue repair process by producing and releasing several active anti-inflammatory and immune molecules (proteins, nucleic acids, lipids) (Caplan 2015; Caplan and Correa 2011; Kean et al. 2013; Vizoso et al. 2017). In other words, in target repair areas, MSCs can release cytokines, growth factors, and chemokines, and help regenerate cartilage by creating a suitable regenerative microenvironment and directing endogenous stem cells to the lesion area (Yang et al. 2020). In the process of differentiation, these cells can produce various ECMs such as collagen (Cols), fibronectin, proteoglycans, and glycosaminoglycans (GAGs), etc. that are necessary to restore cartilage function (Najar et al. 2020; Zha et al. 2021). It should also be noted that all the above explicitly refer to the secretory activity of MSCs as adaptive agents in response to microenvironmental signals, rather than not cells that are merely directly involved in differentiating and replacing the original damaged tissue. These features initially introduce mesenchymal stem cells as an alternative cellular tool with great impact in the field of reconstructive medicine, especially tissue engineering.

17.8.1 MSCs as a Therapeutic Tool or Target

Given the potential therapeutic benefits and following the tissue engineering perspective, MSCs have received a great deal of attention as a therapeutic tool or target. As extensive clinical trials have shown, stem cell therapy, especially MSCs, is a practical strategy in the treatment of cartilage damage that can be differentiated into cartilage cells by supporting cartilage factors or scaffolds to repair damaged cartilage tissue (Hurst et al. 2010; Mithoefer et al. 2009). In general, for cartilage regeneration, access to the source of MSCs can be endogenous or exogenous.

17.8.1.1 Endogenous MSCs

The endogenous method uses the common technique of microfracture surgery, which is more effective for damage in the early stages of cartilage (Mithoefer et al. 2009; Solanki et al. 2021). This technique is a top priority for most orthopedic surgeons for cartilage repair due to its simple one-step technology, limited invasiveness compared to other methods, and its success in relieving pain with a high percentage (Solanki et al. 2021). In this procedure, to stimulate cartilage

regeneration, several holes are drilled in the cartilage bone by the surgeon to discharge BM-MSCs, cytokines, and depleted platelets from the marrow (Solanki et al. 2021; Hayashi et al. 2018). Histological evaluation of the initial changes in cartilage units after microfracture surgery shows that repair due to endochondral ossification occurs deep in the microfracture holes. Also, endochondral ossification can activate osteoclasts and stimulate cartilage regeneration, resulting in cartilage to regenerate earlier than subcutaneous bone (Hayashi et al. 2018). Many studies emphasize that, in the microfracture method, cartilage degeneration can be delayed regardless of the size of the lesion. This technique is also considered by the Food and Drug Administration (FDA) as an excellent prognosis in the treatment of small cartilage injuries (Gudas et al. 2013). However, some studies suggest the formation of relatively unstable fibrous tissue instead of cartilage, indicating that postoperative microfracture microenvironment has not been able to induce BM-MSCs differentiation properly (Gudas et al. 2013; Goyal et al. 2013).

17.8.1.2 Exogenous MSCs

To obtain MSCs exogenously, they are obtained through other mesodermal tissues of the same host (Lee et al. 2019). At present, AT-MSCs and peripheral blood-derived mesenchymal stem cells (PB-MSCs) have been extensively studied in both surgery and injection procedures (Reissis et al. 2016). Surgical incision or intra-articular injection is implanted into the joint to repair small cartilage and the choice of treatment depends mainly on the patient's condition and the specific pathology of the cartilage (Medvedeva et al. 2018). However, according to several clinical trials (Reissis et al. 2016; Wakitani et al. 2002; Davatchi et al. 2016), this approach seems appropriate only for cartilage degeneration in patients with OA (Lee et al. 2019). Traditionally, as mentioned earlier, the reason for this type of therapeutic use of these cells is their ability to differentiate into cartilaginous lineage. In the process of cartilage repair, we face various issues related to the separation and manipulation of MSCs, which can be selected as the most appropriate tissue source and delivery route to the cartilage lesion, and we need to overcome these challenges.

17.8.2 Combination Therapies with MSCs

Several combined therapeutic approaches have been proposed to aid this type of differentiation. The use of induction chemical stimuli, small molecular drugs, inducers, and biomechanical agents, and three-dimensional cultures (engineered scaffolds) have been noted (Chandran and Goel 2012; Buhrmann et al. 2010).

17.8.2.1 Small Molecular Drugs

In MSC-based cartilage regeneration, small molecular drugs have unique advantages over traditional growth factors. Poor induction of the immune response due to their very small molecular size, significant reduction in manufacturing costs, and the risk of cross-contamination of species compared to protein-based growth factors are the advantages of these drugs. Numerous small molecular drugs have been made to

repair cartilage, including kartogenin (KGN) and various natural bioactive compounds (Huang et al. 2020). One of the most common agents is KGN, which can induce the production of chondrocyte MSC in a dose-dependent pattern, as well as improve the production of cartilage-related proteins in mesenchymal stem cells, including Col II and aggrecan (Huang et al. 2020). One study showed that BM-MSCs pretreated with KGN were more effective than normal BM-MSCs in forming cartilage matrix and delaying cartilage deterioration (Huang et al. 2020).

Also, in a study by Spakova et al. (Spakova et al. 2018), KGN was introduced as a chondrogenic promoter of BM-MSC stem cells in the cartilage regeneration process, which after performing histological analysis and scanning electron microscopy (SEM), the effectiveness of KGN in the process of cartilage regeneration was identified (Spakova et al. 2018).

Curcumin and Resveratrol extracts also showed great potential in inducing MSC cartilage differentiation. The high ability of curcumin to inhibit the secretion of inflammatory cytokines in arthritis is well established and helps the MSC regenerate cartilage (Chandran and Goel 2012). Because proinflammatory cytokines interfere with mesenchymal stem cell growth, conventional MSC implantation therapies in cartilage or surrounding tissue are ineffective for treating OA and RA. These inflammatory cytokines are prevented from entering the cartilage environment by curcumin (Buhrmann et al. 2010). Therefore, it facilitates the cartilage formation of mesenchymal stem cells by improving AT-MSC perfusion. Curcumin treatment improved Col II production and proteoglycan synthesis, suggesting that curcumin therapy may support cartilage repair by modifying the AT-MSC inflammatory microenvironment in the joint (Buhrmann et al. 2010).

Like curcumin, resveratrol has an immunomodulatory and anti-inflammatory role (Saiko et al. 2008) and ensures the process of cartilage differentiation in the inflammatory microenvironment by protecting the MSCs and inhibiting the apoptotic signal. In addition, studies report the antiaging properties of resveratrol for the treatment of age-related cartilage disease. The results of studies by Csaki et al. also showed that resveratrol protected BM-MSC-derived cartilage cells from inflammatory agents (Csaki et al. 2008; Shakibaei et al. 2007; Lei et al. 2008).

17.8.2.2 Growth Factors

In the study of cartilaginous potential of growth factors in various clinical trials, bone morphogenetic proteins (BMPs) have shown attractive roles in MSC-based therapies for cartilage regeneration. BMPs activate the cartilage repair process by stimulating endogenous mesenchymal stem cells in the damaged area and boosting MSC cartilage differentiation. For example, BMP2 and BMP7 can cause MSCs to differentiate into cartilage (Kwon et al. 2016; Kangari et al. 2020b). In a study by Dorman et al. (Dorman et al. 2012), the effects of BMP treatment on undifferentiated MSCs investigated. In the early days after BMP treatment, morphological changes from MSCs occurred and cell counts increased, indicating induction of mesenchymal stem cell proliferation and differentiation by BMPs (Dorman et al. 2012). The results of a study by Grand et al. (Grande et al. 2003) also showed that transfected mesenchymal stem cells dramatically improved the quality of repaired tissue, suggesting a

combination of BMPs and MSCs as a potential treatment for cartilage damage. Another crucial growth factor for regulating MSC differentiation in the process of cartilage repair is TGF- β , which can promote the formation of MSC cartilage (Goldring 2006; Facchini et al. 2006). Potential effects of this growth factor on improving functional cartilage differentiation of BM-MSC mesenchymal stem cells have been reported. It also reduces cartilage cell hypertrophy (Kim et al. 2012). Insulin-like growth factor (IGF) is an important cartilage-inducing factor that increases the production of cartilage-related matrices (Uebersax et al. 2008; Zhao et al. 2021). From two ligands of the IGF family, IGF-1 is considered as an essential medium for cartilage homeostasis due to stimulation of proteoglycan synthesis (Uebersax et al. 2008). In addition, improved GAG production by IGF-1 differentiates BM-MSC mesenchymal stem cells into cartilaginous phenotypes (Ikeda et al. 2017). Also, fibroblast growth factor (FGF), especially its ligands, plays a role in strengthening the MSC to repair cartilage damage and can maintain a different cell status (Cleary et al. 2015; Cheng et al. 2012). FGF-2 could enhance MSC amplification and subsequent cartilage differentiation with the Wntless (Wnt) signal. Treatment of BM-MSCs with FGF-2 increased the production of cartilage-related components during the chondrogenesis process (Cheng et al. 2012; Huang et al. 2018).

17.8.2.3 Biomechanical Factors

Because cartilage is a carrier tissue and plays an important role in diarthrodial joints, physical loading is important in maturation and maintenance of its phenotype (Wei and Dai 2021). In MSC-based combination therapies, cartilage regeneration can be enhanced with physical and biomechanical stimuli prior to MSC stimulation, which provides a unique combination of stimulants in cartilage formation and repair. The biomechanical properties of cartilage depend on the composition and order of the ECMs (Wei and Dai 2021; Zhuo et al. 2012). Hydrostatic pressure, shear, and density are examples of articulated biomechanical properties. Therefore, mechanical stimuli that can be used to repair cartilage include pressure, hydrostatic pressure, and shear stress. In a study on embryonic bud cartilage, it was found that static compression could increase cartilage formation by positively regulating the expression of Col II, Sox9, and aggrecan (Vágó et al. 2021; Takahashi et al. 1998). This was confirmed in another study on the application of intermittent hydrostatic pressure (Takahashi et al. 1998; Miyanishi et al. 2006). In addition, the construction and availability of bioreactors greatly contribute to the application of complex physical loads (hydrostatic pressure and compression) in MSCs (O'Connor et al. 2013). In one study, the effect of multiple bioreactors to mimic these biomechanical properties in vivo was investigated, and the use of multimodal bioreactors improved the cartilaginous conditions of MSCs (Grad et al. 2011). The effect of dynamic compression on MSC cartilage formation has been extensively investigated in several papers and reviews (Huang et al. 2005; Fahy et al. 2018). For example, in a study on the expression of Col II, Sox9, and aggrecan, Huang et al. (Huang et al. 2005) reported a positive expression result following dynamic compression on rabbit BM-MSCs. In the study combining cyclic compression with incision, it was found that even

without the activation of exogenous growth factors, an increase in GAG and Col II deposition was observed in addition to an increase in BM-MSC differentiation into chondrocytes (Schätti et al. 2011). As a result, the potential secretory activity of BM-MSCs can be induced by physical stimuli (Schätti et al. 2011). As Gardner et al. (Gardner et al. 2017) confirmed that the increase in endogenous production and secretion of TGF- β 1 occurred following cleavage and compression (Gardner et al. 2017). In addition, cartilage formation for cartilage repair can be enhanced by combining scaffolding with mechanical stimuli (which mimic and simulate a native cartilage microenvironment) (Sawatjui et al. 2018). In some studies, improved production of cartilaginous matrices, Aggrecan, and Col IIA1 in BM-MSC and cartilage cells was reported. This positive result was achieved by the microenvironment provided by porous scaffolds based on silk fibroin (SF) and SF with gelatin/chondroitin sulfate/hyaluronate (SF-GCH) along with compression (Sawatjui et al. 2015, 2018). In such conditions, the spatial control of the local biochemical and mechanical properties of the scaffold as well as the external dynamic pressure can cause the cell deformation of the scaffold. As Neven et al. by creating a hydrogel scaffold with layers of different stiffness, produced different strains on it (Steinmetz et al. 2015). So, this finding suggests that appropriate mechanical stimuli are potent regulators of MSC differentiation and may act as a potential stimulus for the treatment of cartilage damage. In general, access to the source of MSCs can be endogenous or exogenous for cartilage regeneration.

17.9 Clinical Trial

Using autologous cells, mostly chondrocytes, some of which may have been chondroprogenitors or stem cells, Brittberg et al.'s approach to repairing cartilage damage was the first application of cell therapy in an orthopedic setting (Simon and Jackson 2018; Armiento et al. 2019). So far, several clinical trials have utilized human stem cells to treat diseases and injuries related to cartilage (Table 17.2).

Furthermore, multiple clinical researchers from different regions of the world have reported the effectiveness of SC administration in treating OA patients. Patients' perspectives have changed dramatically as a result of clinical trials using stem cells to treat diseases and cartilage injuries, such as osteoarthritis, joint diseases, and articular cartilage defects. MSCs were one of the most significant stem cells used as cartilage defects' therapies throughout clinical studies. The majority of the 132 trials, which were conducted between March 2000 and April 2022, used MSCs of the same type, while a few also used MSCs from various tissue origins (Musumeci et al. 2015). In these clinical trials, MSCs often originate from bone marrow and adipose tissue (Musumeci et al. 2015). For example, using an intra-articular injection of culture-expanded adipose tissue-derived stem cells, a small sample of patients with knee osteoarthritis demonstrated considerable improvement at 6 months, with more relevance in knees with a Kellgren-Lawrence (KL) grade 2 or 3 compared to KL grade 4 knees (Lespasio et al. 2017).

Table 17.2 Major clinical trials of stem cell therapy for treating diseases associated with cartilage defects

Clinical trials. Gov identifier and clinical trial phase	Ages eligible for study	Number of enrolled participants or patients	Stem cell type	Adverse event	Outcome	Reference
(NCT03825133) IV	Over 60 years	111	Bone marrow-derived stem cells	No adverse effect observed	No significant differences in outcome or pain scores were observed among groups	Dulic et al. (2020)
(NCT02641860) I/IIa	18 – 70 years	18	Adipose-derived stem cells	No adverse effect observed	Significant differences were observed in quantitative T1rho, T2, T2star, R2star, and ADC measurements in patients of three dose groups. significant reduction in WOMAC, and SF-36 scores observed	Zhao et al. (2019a)
(NCT02162693) II	18– 70 years	25	Adipose-derived stem cells	Adverse effects were mild and moderate	Significant improvements were observed in joint function, pain, quality of life, and cartilage regeneration in a period of 12 months	Lu et al. (2019)
(NCT02658344) II	18 years and older	24	Adipose-derived stem cells	No serious adverse events were observed	Satisfactory functional improvement and pain relief for patients with knee osteoarthritis were observed	Lee et al. (2019)
(NCT02580695) I/II	18– 70 years	26	Umbilical cord-derived mesenchymal stromal cells	No severe adverse events were reported	UC-MSC treatment is safe and superior to active comparator in knee OA at 1-year follow-up	Matas et al. (2019)
(NCT02641860) I	18– 70 years	18	Adipose-derived stem cells	No severe adverse events were reported	Improved WOMAC scores at 1 year	Zhao et al. (2019b)
(NCT02351011) I/II	40– 65 years	12	Bone marrow mesenchymal stromal cells	No serious adverse events were reported	Significant improvements in patient-reported outcome measures at 12 months	Chahal et al. (2019)

(continued)

Table 17.2 (continued)

Clinical trials. Gov identifier and clinical trial phase	Ages eligible for study	Number of enrolled participants or patients	Stem cell type	Adverse event	Outcome	Reference
(NCT01733186) I/II	18 years and older	12	Umbilical cord blood-derived mesenchymal stem cells	No serious adverse events were reported	Improved IKDC, pain, KOOS scores (less pain, less symptoms, and a higher level of physical functioning)	Not published
(NCT02674399) II	22–60 years	26	Adipose-derived stem cells	No serious adverse events were reported	Improved WOMAC, VAS pain scores at 1 year (less pain and stiffness and better physical function)	Not published
(NCT02784964) I/II	40–80 years	57	Autologous mesenchymal stem cells	No related serious adverse events were reported	Significant decreases at Week 4 compared to HA group in WOMAC total scores, stiffness scores, functional limitation scores observed	Chen et al. (2021)
(NCT02365142) I/II	40–80 years	38	Bone marrow mesenchymal stromal cells	No related serious adverse events were reported	Improvement at the end of the follow-up in VAS and WOMAC score	Lamo-Espinosa et al. (2020)
(NCT03589287) I/II	40 years and older	18	Bone marrow mesenchymal stromal cells	No related serious adverse events were reported	Overall improvement in pain and symptoms and reduces synovial inflammation	Not published
(NCT01504464) II	18–65 years	40	Bone marrow mesenchymal stromal cells	No related serious adverse events were reported	MSC-based therapy significantly improved the KOOS at the 12-month follow-up and relieved pain	Emadedin et al. (2018)
(NCT01207661) I	18–65 years	6	Bone marrow mesenchymal stromal cells	No severe adverse events were reported	All patients exhibited therapeutic benefits such as increased walking distance, decreased visual analog scale (VAS), and total Western	Emadedin et al. (2015)

(NCT00225095) I/II	18– 60 years	60	Bone marrow mesenchymal stromal cells	No related serious adverse events were reported	Ontario and McMaster Universities OA Index (WOMAC) scores	Vangsness Jr et al. (2014)
(NCT02123368) I/II	50– 80 years	30	Bone marrow mesenchymal stromal cells	No related serious adverse events were reported	The beneficial effect of MSCs were both significant and sizeable, and it can be achieved with a single injection of cells, and the effect is perdurable for years	Lamo- Espinosa et al. (2021)

In addition, at short-term follow-up, intra-articular injection of autologous ATSCs or adipose-derived stromal vascular fractions (ADSVFs) in patients with knee OA showed exceptional clinical effectiveness and safety (Palazzo et al. 2016). Using BM-MSC was first described by Wakitani in 23 OA patients (Collins et al. 2019). He discovered that although clinical improvements were not significantly different across groups, arthroscopy and histological scores were improved in the MSC-treated group at roughly 42 weeks (Collins et al. 2019). Another interesting research report is that of Maheswari et al. describing the intra-articular administration of allogeneic, off-the-shelf, bone marrow-derived, pooled, mesenchymal stem cells in patients with grade II and III osteoarthritis of the knee. The findings of this study showed that the application of these stem cells in patients with grade II and III osteoarthritis of the knee provided long-lasting pain and stiffness relief, improved physical function, prevented worsening of cartilage quality, and improved cartilage volume for at least 12 months after treatment (Li et al. 2017). While X-rays showed no worsening of arthritis and MRIs showed a constant filling of the chondral defects by the implant, significant improvements were noted in Lysholm Knee Scoring Scale (Lysholm score) and Knee Injury and Osteoarthritis Outcome Score (KOOS) in patients using BiCure[®] orthoMSCp, a three-dimensional scaffold made of cultured stem cells (Li et al. 2017). In addition, by application of an autologous chondrocytes' combination and allogeneic BM-MSCs, the IMPACT trial with Clinical Trial identifier NCT02037204 in ClinicalTrials.gov, evaluated the safety and efficacy of a single-stage treatment for localized cartilage lesions in the knee (Boehme and Rolaufts 2018). The 5-year result analysis suggests that, compared to baseline, most of the patients showed improvement in the KOOS and all of its subscales statistically significant and clinically relevant (Boehme and Rolaufts 2018). Currently enrolling participants are in the follow-up 60 patient randomized placebo-controlled IMPACT2 experiment (<http://clinicaltrials.gov>: NCT04236739) (Medvedeva et al. 2018).

Furthermore, amniotic tissues, peripheral blood, dental pulp, placental, and the blood from the umbilical cord were employed to make the MSCs for the clinical studies. For example, in the study by Song et al., mesenchymal stem cells (hUCB-MSCs) generated from human umbilical cord blood were used to treat medial compartment (MC) osteoarthritis of the knee. The findings of this investigation showed that in individuals older than 60 with MC osteoarthritis, hUCB-MSC implantation successfully restored cartilage and produced good clinical effects (Roseti et al. 2019). In a case series study, the effectiveness of implanting allogenic umbilical cord blood-derived mesenchymal stem cells to treat knee osteoarthritis was evaluated after a 2-year follow-up. When compared to the preoperative scores after 1- and 2-years following surgery with hUCB-MSC implantation, the mean (standard deviation) visual analog scale (VAS), the Western Ontario and McMaster Universities Arthritis Index (WOMAC), and International Knee Documentation Committee (IKDC) ratings were significantly increased (Bianchi et al. 2017). As a therapeutic agent for cartilage regeneration, CARTISTEM[®], an allogeneic umbilical cord blood-derived MSC product coupled with sodium hyaluronate, has been employed in numerous research and trials, including NCT01041001,

NCT01626677, NCT01733186 (<http://clinicaltrials.gov>) (Jiang et al. 2020). The VAS and IKDC scores were improved at 24 weeks after the infusion of CARTISTEM[®] in a clinical trial for safety and proof-of-concept with 7 years of extended follow-up (De Bari and Roelofs 2018). Over a 7-year period of follow-up, the better clinical outcomes were stable. At 1 year, the histology findings revealed cartilage that seemed hyaline. The regenerated cartilage was still present in the MRI after 3 years. There were only five mild to moderate side effects that developed throughout treatment. Over a 7-year period, there were no reports of osteogenesis or cancer. The results of a multicenter randomized clinical trial and extended 5-year clinical follow-up demonstrated that CARTISTEM[®] implantation versus microfracture for large, full-thickness cartilage defects in older patients could result in enhanced cartilage grade at second-look arthroscopy and provided more pain relief and function up to 5 years compared with microfracture (Li et al. 2018). Turajane et al. used intra-articular implantation of autologous activated peripheral blood stem cells for OA of the knee (Ma et al. 2018). This study's findings revealed that, compared to controls, the autologous activated peripheral blood stem cells groups' Total WOMAC scores improved statistically significant at 6 and 12 months. There were no significant negative complications (Ma et al. 2018). In addition, the research study conducted by Nabavizadeh et al. demonstrated that the combination of synovial membrane-derived MSCs (SMMSCs)/secretome/PRP had a significant impact on the contents of glycosaminoglycans (GAGs) and collagen II and the preservation of articular cartilage (Yamanaka 2020).

Current therapeutic approaches of local cartilage lesions and OA center on pain relief and inflammation reduction without significant long-term effects. As the other authors stated, and as we concur, long-term follow-up has thus far shown possible disadvantages including the MSCs tendency to generate hypertrophic chondrocytes and bone instead of high-quality hyaline cartilage during the chondrogenesis following cell therapy. This suggests that additional efforts are required to recognize this treatment as a golden standard of care.

17.10 Horizons and Challenges Ahead

Despite recent promising advances, multiple challenges remain that prevent the clinical application of hESC-based cell replacement therapies in patients. One major obstacle is the immune rejection of cells derived from hESCs due to allogeneic antigens (mismatched major human leukocyte antigens (HLAs)), after transplantation to patients. Application of immune suppressors may not be a good approach because of serious risks of cancer and infection (Fu and Xu 2012). The risk of teratoma formation is another obstacle that could be eliminated by developing approaches to discard undifferentiated hESCs from their derivatives (Choo et al. 2008; Tan et al. 2009).

However, despite the ability of MSCs to differentiate into cartilaginous lineages, sometimes the final phenotype is in the form of endochondral bone formation instead of cartilage cells (especially in the joint area, which indicates the formation of bone

within the cartilage), which, of course, can correct (Somoza et al. 2014). As a result, the clinical use of mesenchymal stem cells for cartilage repair, especially in the case of large lesions, is still questionable (Filardo et al. 2013). Similarly, from a clinical point of view, there are reports of impaired tissue formation and problems with fusion with surrounding tissue and subcutaneous bone (De Girolamo et al. 2016; Mamidi et al. 2016). In addition, the recent discovery of human skeletal stem cells (hSSCs) and their vital phenotypic adaptations during cartilage specialization pose challenges for the use of MSCs in articular cartilage repair (Tichy and Mourkioti 2018). In fact, the study of adult stem cells in tissues that lack a vascular source with their limited ability to regenerate is a field of research that has been studied to some extent and is receiving increasing attention (Zakrzewski et al. 2019; Majka et al. 2017).

Remarkably, a mixed population of human cartilage cells and precursor cartilage (collectively called cartilage cells) exhibiting stem-like phenotypic and immunophenotypic flexibility has been reported in healthy and degenerated articular cartilage (De Luca et al. 2019), compared to benign mesenchymal stem cells from adipose tissue or bone marrow, these mesenchymal stem cells have higher cartilage, higher basal secretion of growth factors and cytokines, especially after inflammatory priming, and behavior, showed significant safety modulators (De Luca et al. 2019). Specifically, at baseline, a combined population of cartilage cells and precursor cartilage showed the highest rates of cartilage secretion, angiogenesis, and premitogenic growth factors. As a result, the direct use of mesenchymal stem cells for the engineering of articular cartilage tissue, utilizing their cartilaginous differentiation, can be used primarily to treat focal and osteochondral cartilage lesions.

Albeit the proliferation rate (Kang et al. 2015) and chondrogenic differentiation potential of iPSCs are higher (Li et al. 2016; Ko et al. 2014) than MSCs, there are still several limitations that should be overcome before translation to the clinic. Due to high price of development and transplantation of patient-specific autologous iPSC, allogeneic therapy could be another potential candidate but the immune rejection should be considered as well (Zhao et al. 2011).

Similar to MSCs, there is still doubts in the potential of regenerated cartilage induced by iPSCs to maintain mechanical and functional features of native articular cartilage. In addition, it is possible that iPSCs form low-quality cartilage tissue (Nam et al. 2017; Ko et al. 2014). Safety issues including reactivation of pluripotency in iPSCs or iPSC-derived chondrocytes (Lee et al. 2013) and increased risk of teratoma formation resulted from retrovirally transduced iPSCs (Okita et al. 2007) should be considered. Eventually, creating optimal phenotyping of chondrogenic committed iPSCs and highly efficient differentiation protocols is needed before translation to clinical use (Lach et al. 2022). Various studies have introduced novel approaches to develop iPSCs with a lower risk of teratoma formation (Garreta et al. 2018; Jacquet et al. 2013; de Almeida et al. 2013).

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Validation of Tissue-Engineered Constructs: Preclinical and Clinical Studies 18

Maryam Talebi Jouybari, Nesa Fani, Shahrbanoo Jahangir, Fatemeh Bagheri, Reihaneh Golru, and Leila Taghiyar

Abstract

The weak self-regeneration ability of articular cartilage following injury and the unacceptable results of traditional clinical necessitate innovative tissue-engineering strategies to create a hopeful therapeutic approach for articular cartilage repair. The strategies used for tissue engineered articular cartilage include cell-based-engineering, scaffold-free, and cell-free approaches. Using cells sources such as autologous

Maryam Talebi Jouybari and Nesa Fani contributed equally to this work.

M. Talebi Jouybari

Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Department of Developmental Biology, University of Science and Culture, Tehran, Iran

N. Fani

Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

SinaCell Research and Production Co., Pardis, Iran

S. Jahangir

AO Research Institute Davos, Davos, Switzerland

F. Bagheri

Department of Biotechnology, Faculty of Chemical Engineering, Tarbiat Modares University, Tehran, Iran

R. Golru

Faculty of Biological Sciences, Alzahra University, Tehran, Iran

L. Taghiyar (✉)

Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

e-mail: leilataghiyar@royaninstitute.org

chondrocytes and stem cells is prevalent in cell-based engineering and scaffold-free strategies for cartilage defects. Despite the limitations in utilizing autologous chondrocytes and the encouraging outcomes of clinical and preclinical studies using stem cells, the first commercial cell-based tissue-engineered products approved by the food and drug administration (FDA) used autologous chondrocytes. As another alternative, cell-free strategies such as using extracellular vesicles (EVs) has recently entered clinical settings and, despite promising preclinical results, requires further investigation and knowledge. One of the newly discovered strategies is the scaffold-free strategy, which is a challenging method that needs further investigation. In this chapter, the techniques and challenges in the tissue engineering method for articular cartilage restoration are reviewed. By examining the mentioned new approaches of cartilage tissue engineering, using scaffolds and various seed cells, the details of the clinical application status of articular cartilage-engineering will be explained. In addition, evaluation methods used in preclinical and clinical studies as well as cellular products used for articular cartilage repair that have received FDA approval will be reviewed.

Keywords

Articular cartilage · Cell-based-engineering · Cell-free-products · Scaffold-based product

18.1 Introduction

Arthritis, more specifically osteoarthritis (OA) is the most common cause of disability in older people, resulting in a substantial burden on healthcare systems. The Osteoarthritis Research Society International (OARSI) White Paper described OA as a serious disease for prevalence and subsequent economic burden (OARSI White Paper 2018). Due to the cartilage's avascular nature and scarcity of cells, the intrinsic capacity of articular cartilage for healing after an injury is limited, which makes it even more challenging to develop regenerative medicine repair strategies (O'Connell et al. 2017; Kwon et al. 2019).

By 2030, the number of adults in the United States suffering from OA are expected to rise to 67 million (Zhao et al. 2019; Murphy and Helmick 2012). Till now, numerous clinical approaches have been presented depending on the condition of the patient and the degree of cartilage damage. Even though some of the results of these treatments were positive, they were proven to be alleviating and not curative, which can deter their long-term clinical application. For instance, cartilage produced from the microfracture method is often composed of type I collagen (representative of fibrocartilage), which is biochemically and biomechanically inferior to hyaline

cartilage. On the other hand, allograft use presents limitations regarding disease transfer and immune rejection.

Cell-based therapeutic approaches is a promising procedure for large cartilage defects with durable and effective repair in studies lasting as long as 9 years, such as autologous chondrocyte implantation (Peterson et al. 2000). The drawbacks of using chondrocytes such as requiring invasive operations, the low number of chondrocytes, cell dedifferentiation during expansion, and fibrocartilage formation rather than hyaline cartilage in the defect site limit the efficiency of this method (Roberts et al. 2009).

Overall, there is currently no satisfying clinical technique that is widely accepted to stimulate cartilage regrowth and long-term replacement for native cartilage. In response to this need, manufacturing neo-tissues through tissue engineering methods is indicated for patients before total joint arthroplasty. To date, many different strategies have been recommended and applied for cartilage repair and regeneration, (1) cell-based strategies, a combination of numerous types of growth factors, drugs, and extracellular vesicles with cells or scaffolds, (2) scaffold-free strategies, and (3) cell-free strategies.

At present, the efficacy of implanted constructs for restoring the function of the tissue can only be confirmed by *in vitro* experiments as well as implantation in preclinical animal models. Since there are limitations to using animals as an efficient model, it would be ideal to develop technologies that have high diagnostic power but are noninvasive, specially with the ability to provide a real-time and obvious picture of the defect healing and biomaterial-host interactions (Trachtenberg et al. 2015).

Over the past 20 years, despite the existence of emerging strategies and approved commercial cell-based or cell-free products, articular cartilage regeneration and functional recovery remain one of the main challenges in clinical and experimental settings. Investigating the innovative treatment approaches for cartilage tissue repair, their outcomes in preclinical studies, and the limitations of utilizing them in clinical studies, as well as the restriction of methods for evaluation of their results, seem to answer the unsolved problems in articular cartilage regeneration. Therefore, this chapter will focus on the latest therapeutic strategies, preclinical studies, and various methods for investigating clinical and preclinical outcomes. As well, the latest achievements of commercial products in the field of cartilage tissue repair will be introduced and evaluated.

18.2 Present Status for Articular Cartilage Repair

18.2.1 Traditional Surgical Regeneration Techniques

There are various treatment choices for articular cartilage defects, including arthroscopic debridement, transplantation of whole osteochondral allografts (OCA) and osteochondral autografts transplantation (OAT), and mosaicplasty (Makris et al. 2015; Kraeutler et al. 2020), as well as, marrow stimulation, such as microfracture,

can treat the majority of smaller sizes (about 0.6–2 cm²) and local chondral defects as the initial line of treatment (Saris et al. 2014; Memon and Quinlan 2012; Marcacci et al. 2013). Microfracture by mechanical inspiration stimulates the autologous bone marrow and triggers a regenerative response from the mesenchymal stem cells (MSCs) in the bone marrow to fill the gap in the subchondral bone (Steadman et al. 2010). Clinical evidence of microfracture shows success in decreasing pain and repairing small focal chondral holes in the knee (3.6 cm²). Long-term follow-up results, however, demonstrate that integral cartilage does not form, tissue is primarily made of fibrocartilage, and that failure rates increase after 2–5 years (Gao et al. 2019; Becher et al. 2019; Solheim et al. 2016). In many cases, optimal treatment selection is influenced by several variables, including the patient's age, defect grade, and location. For instance, younger patients (about 40 years old) have shown more improved outcomes than older patients. The lesion size is the main feature in the selection of treatment for local cartilage defects. So, other techniques such as osteochondral allografts are better choices for extra-large defects.

18.2.2 Osteochondral Transplantation (OT)

The first OT transplantation was reported in 1985 by Yamashita for restoration of knee cartilage defects (Yamashita et al. 1985). The Fresh osteochondral allograft (single-stage surgery) or autograft transplantation (double-stage surgery) is an operation in which a small osteochondral cylinder is removed from the patient's nonweight-bearing articular surfaces like the femoral trochlear and transplanted into articular cartilage defects, identified as mosaicplasty. The advantages of OT are being simple operation, having no need for immune rejection (in autograft), and defect being filled by hyaline cartilage (Haber et al. 2019). The outcomes of long-term follow-up to 17 years showed 70–90% had normally good to excellent effects based on the location of defects (Harris et al. 2010). The key difficulties are the limitation of graft sources and secondary defects at donor sites, suitability for patients with small defect sizes and younger than 45 years with good physical condition and focal lesions. Also, in many cases, the osteochondral column cannot perfectly fit into the whole defect zone, which may lead to pressure concentration and surgical failure in the long-term. Therefore, considering the risk-benefit ratio of OT, it may not be a complete clinical application for cartilage treatment.

18.2.3 Autologous Chondrocyte Implantation (ACI)

In chondral defects, advanced methods can regenerate natural cartilage particularly by using chondrocytes from the patient's cartilage (autologous chondrocytes). Chondrocytes from the user's own body are the main component in these therapeutic cell-based cartilage repair therapies (Ye et al. 2014; Rath and Tingart 2017). Significantly, sourcing autologous cells predicated on the patient serving as both the donor and the recipient poses few immunological obstacles. The first cell

treatment for articular cartilage using chondrocytes was carried out by Lars Peterson in 1987 and he later reported first his cohort study in 1994. At first, the autologous chondrocytes are isolated from a patient's healthy, and low-weight-bearing space, thereafter, cells are cultured and grown *ex vivo*, and then expanded cells are injected into the cartilage defect under an already sutured periosteal flap (Kon et al. 2012). ACI offers more consistent and enhanced pain relief and mobility results during 5-year checkups compared to microfracture. Later an enhanced generation of ACI (first generation) used an external scaffold such as collagen I or III as well as hyaluronic acid to fix the constructs in the defect site, that showed more effectiveness. Utilization of this therapy with porcine collagen matrix-supported autologous cultured chondrocytes (MACI) (Kon et al. 2012), has achieved FDA approval in 2016 (Hambly 2011). This collagen membrane supports chondrocyte properties during culture and keeps cells during transplanting, the defect site (Kon et al. 2012; Jones et al. 2008). Short-term clinical follow-ups of 2 years reported that 75% hyaline-like tissue filled the defects, and 15-year follow-ups revealed that the International Knee Documentation Committee (IKDC) (Barber-Westin and Noyes 2010), Lysholm (Kocher et al. 2004), scores from Tegner activity (Hambly 2011), and preoperative baselines are contrasted. Although the superiority of Maboutn to ACI is still debatable in 2-year randomized trials following-up, the IKDC and Tegner activity ratings showed no discernible improvements. ACI reported somewhat improved International Cartilage for MACI compared to ACI. Lysholm functioning scores (Makris et al. 2015; Kon et al. 2012; DiBartola et al. 2016) and international cartilage repair society (ICRS) scores are two examples.

Although the follow-up of patients in clinical studies using autologous chondrocytes has shown acceptable outcomes in the long-term, there were several restrictions including donor site lesions, tissue availability, and loss of chondrocyte phenotype after *ex vivo* expansion or fibrocartilage formation (Minas et al. 2016). Stem cells with unique properties, on the other hand, have been considered a potential therapeutic goal in regenerative medicine when it came to cartilage and osteochondral lesions treatment.

18.2.4 Allogeneic and Autologous Stem Cells Implantation

MSCs are emerging as accessible and safe cell sources in regenerative medicine. The paracrine effects, immunomodulatory, and homing properties of these cells have elicited significant interest in regenerative treatments. Using allogeneic MSCs without the risk of rejection give greater control over cell quality with better accessibility and construction of "off-the-shelf" products (Thorpe et al. 2021). The MSCs obtained from adipose tissues, placenta cord, umbilical cord, and bone marrow have been extensively investigated for use in cartilage tissue regeneration in preclinical (Sampath et al. 2021; Wang et al. 2021; Han et al. 2021) and clinical studies such as the Wang study that injected human umbilical cord MSCs in 36 patients with moderate or severe degenerative knee osteoarthritis. The intra-articular injection of sodium hyaluronate was used in the control group. During 6 months follow-ups, the

Lysholm score, and WOMAC score showed remarkably better results in the cell-treated group in comparison to preoperative scores (Wang et al. 2016). In a double-blind, placebo-controlled clinical trial, khalifeh soltani et al. injected either $0.5\text{--}0.6 \times 10^8$ allogenic placenta-derived MSCs or normal saline into 20 patients with knee OA. At 24 weeks of follow-up. They observed an improvement in chondral thickness in almost 10% of the whole knee joint area in the treatment group (Soltani et al. 2019).

The satisfactory results of using these cells in clinical trial studies have led to the commercialization of several cellular products. For instance, Park et al. used a commercial product (composed of culture-expanded allogeneic human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) and hyaluronic acid hydrogel [CARTISTEM[®]]) for seven cases with ICRS grade 4 cartilage defects with 7-years follow-up. The histological findings indicated the formation of hyaline-like cartilage. During follow-up, improved clinical outcomes were constant (Park et al. 2017). A controlled phase 3 clinical trial for CARTISTEM[®] was controlled for 48 weeks, and 5-year outcomes from 89 patients have recently been reported with microfracture. The improved cartilage grade at arthroscopy was seen in the CARTISTEM[®] treated patients versus the microfracture group (Lim et al. 2021). In many cases, MSCs were used in combination with scaffolds for articular cartilage regeneration. Therefore, in the following section, we will discuss more studies that have used engineered cartilage structure.

18.3 Progress Toward Tissue-Engineered Cartilage

18.3.1 Tissue-Engineered Constructs

Tissue engineering (TE) is a multidisciplinary research field incorporating engineering principles with biology science to produce new living tissues to replace damaged tissues and restore their function (Kuo et al. 2006). Cartilage has become an ideal candidate for tissue engineering due to its limited tendency for self-healing (Bhardwaj et al. 2015). Indeed, one of the most promising therapeutic advances in recent years is the TE-based strategy for cartilage regeneration by combining the scaffolds, cells, and/or soluble/mechanical factors (From the American Association of Neurological Surgeons (AANS) et al. 2018). Principally, the TE process for articular cartilage involves several steps: expanding suitable cells in vitro, then seeding or encapsulating them into a scaffold to develop a cellular scaffold structure, and subsequently, implanting the constructs into the tissue defect (Vinardell et al. 2012). Each step of the TE process, had many challenges, such as the choice of cell sources, and appropriate scaffolds (Nerem 2006). Many efforts have been made to answer the question of which cell source is optimal for cartilage TE (Martincic et al. 2021). Chondrocytes initially were used as the ideal cell source but in the long term, disadvantages were shown at follow-ups. The very first approach of autologous MSC implantation was in a rabbit osteochondral defect, which was reported by

Pakistani et al. in the year 1994. These results then were confirmed by other scientists (Wakitani et al. 1994; Caplan et al. 1997).

Scaffolds derived from decellularized extracellular matrix (ECM) as a new generation of natural scaffolds revealed prominent biological properties when it came to providing the native microenvironment to direct the differentiation of stem cells. Numerous studies have proven that ECM-derived scaffolds mimicking nano-fibrous structure and architecture valuably enhance cell-material transport, cell migration, proliferation, tissue regeneration, and integration with the host tissue (Visscher et al. 2021; Shin et al. 2021; Changoor et al. 2021).

Despite the amazing progress that has been made to fabricate natural scaffolds to mimic native bone tissue, these materials could not fully mimic the tissue niche (microenvironment) and mechanical properties of native tissues (Liu et al. 2017). Cell-free and scaffold-free approaches are the two new methods that have become attractive research areas in recent years which will be explained in the following.

18.3.2 Cell-Free Approaches for Cartilage Regeneration

Cell-free biofunctional scaffolds have been widely studied as a promising alternative strategy to promote cardiovascular, bone, cartilage, and meniscus tissue repair. In cell-free techniques, unlike traditional cell-based treatments with a potential for clinical translation, the chemoattractant gradients, bioactive scaffolds, as well as innate chemotactic properties of ECM-based materials are used to build the engineered scaffolds. These scaffolds can release biological signals that are capable of mimicking the complex signaling patterns of endogenous tissue regeneration. For instance, the injuries of meniscus as a fibrocartilaginous structure are popular and challenging to remedy which will naturally lead to the development of OA. Indeed, in meniscal tissue engineering, the cell-free technique may avoid many of the restrictions and dangers of cell-based techniques including choice of suitable cell sources, its expansion in scaffolds, cellular contamination, and an increased risk of disease, as well as surgical duration (Gille et al. 2010). In preclinical experiments, cell-free scaffolds including acellular meniscus extracellular matrix (AMECM), demineralized cancellous bone (DCB), and a combination of AMECM/DCB and poly-glycolic acid used in ovine or rabbit's meniscus animal models, yet. The results of the histological score, MRI, and meniscectomy showed significant improvement in investigational groups in comparison to the control group at both 3- and 6-months follow-ups (Erggelet et al. 2009; Yuan et al. 2016; Guo et al. 2018). In a clinical trial study, Efe et al. used a cell-free collagen type I matrix to treat patients with cartilage damage with 24-month follow-up. The outcomes of the MOCART score and magnetic resonance imaging (MRI) confirmed the complete filling of defects in all cases with a flat surface and wide-ranging integration of the construct with native tissues, at 24 months after implantation (Efe et al. 2012). Clinically, the cell-free techniques stimulate the body's repair mechanisms and this stimulation is done by recruiting the endogenous stem/progenitor cells and getting them to take part in the repair process. Many tissues and organs have a reserve of endogenous stem/

progenitor cells. Following an injury, the stimulation of local stem cells and their recruitment to the defect sites leads to them repairing tissue structure and organ function over time (Zhang et al. 2020; Lim and Son 2017). Therefore, the application of proper stimulation and recruitment of stem cells are key elements to successful cell-free strategies for meniscus or cartilage repair.

Indeed, stimulating local endogenous stem/progenitor cells to migrate into the injury sites is the first and the main step of cell-free approaches. Then, the proliferation, and differentiation of migrated cells as well as cell maturation and return of tissue function will occur (Rennert et al. 2012). Recently, several methods such as the use of specific cell markers like proteoglycan 4 (Qadri et al. 2021) or growth/differentiation factor 5 (GDF-5) (Witoonpanich et al. 2022), chemokines (Park et al. 2015), and platelet-rich plasma (PRP) (Abrams et al. 2013), have shown an appropriate effect on cellular recruitment following cartilage knee lesions.

For instance, the two bioactive molecules E7, and transforming growth factor- β 1 (TGF- β 1) are released in a controlled sequential form. Following that, Mao et al. developed a cartilage-biomimetic silk fibroin (SF)-based scaffold. Indeed, the very first rapid release of E7 during the first few days, and followed by the slow and sustained release of TGF- β 1 for a few weeks, synergistically led to the recruitment of MSCs and promoting in situ cartilage regeneration in a rabbit cartilage defect model (Mao et al. 2022).

Other cell-free approaches are based on natural scaffolds such as MaioRegen and TruFit Plug[®]. MiaoRegen is a 3D and two-layer matrix that mimics the entire osteochondral tissue. The superficial layer contains type I equine collagen and resembles the cartilaginous tissue, while the lower layer is mostly made up of magnesium-enriched hydroxyapatite (Mg-HA), and is in charge of stimulating the subchondral bone structure. Considering the limited clinical studies related to this product, the evidence does not show the superiority of the MaioRegen model. Additionally, long-term follow-up is needed (D'Ambrosi et al. 2019). The TruFit Plug is a biphasic, acellular synthetic scaffold that is made from polylactide-coglycolide copolymer to stimulate cartilage and regenerate subchondral bone. Calcium sulfate is utilized for the bone phase and the infiltrates of cells and growth factors derived from the bone marrow into the plug cause cartilage regeneration. The long-term results showed that TruFit Plug is safe based on postoperative scores such as pain enhancement, function, and MRI results. However, further randomized controlled clinical trials are necessary to confirm TruFit Plug comparison to established treatment methods for osteochondral defects (Di Cave et al. 2017; Kolar and Drobic 2022).

As the cell-based strategies for cartilage repair developed, many tissue engineering methods took advantage of synthetic and natural materials, to provide appropriate 3D constructs for cells and improve their proliferation and differentiation. Also, it effectively delivers cells into cartilage defects. Due to the unsolved challenges of scaffolds, a scaffold-free cell delivery system could be used as an alternative prominent selection because of its ease of development and simplicity of subsequent implantation, and lower cost, without the use of expensive biomaterials.

18.3.3 Scaffold-Free Constructs

Newer cell-free TE strategies for the treatment of the lesion are based on the use of scaffold-free tissue-engineered constructs. Recently, several studies reported the scaffold-free tissue-engineered constructs prepared for the regeneration of some tissues like meniscus, cardiac, bone, and cartilage (Ando et al. 2007; Toratani et al. 2017; Noguchi et al. 2016). For cartilage repair, except for the MSCs, numerous cell sources like fibroblasts, chondrocytes, transgenic cells, embryonic stem cells, and amniotic fluid-derived stem cells have been examined. These candidate cells are being used alone or suspended in components such as polysaccharide gels for cartilage regeneration. For instance, in a preclinical study, a monolayer MSC-based tissue-engineered construct (TEC) was generated *in vitro* for repairing a porcine chondral defect model. *In vitro* analysis of the cell/matrix complex revealed that the basic TEC including collagen I and III, vitronectin, and fibronectin resulted in a high expression of glycosaminoglycan and chondrogenic marker genes. The TEC was then implanted into chondral defects in pigs. After 6 months, chondral defects initiated repair with a chondrogenic-like tissue collagen II expression, and secured biological integration to the adjacent cartilage (Ando et al. 2007). In a clinical study, TEC derived from autologous synovial membrane mesenchymal stem cells (sMSCs) under low oxygen tension made for effective cartilage repair in five patients with symptomatic knee chondral lesions. After *in vitro* TEC production, they were implanted into chondral defects. The long-term follow-up of clinical scores, arthroscopy, and MRI showed that pain, symptoms, daily life activities, sports activity, and quality of life were all remarkably improved 24 months postsurgery. Furthermore, histological analysis confirmed the formation of the structure of hyaline cartilage in filled defects by TEC (Shimomura et al. 2018). Additionally, novel commercial scaffold-free constructs such as NOVOCART 3D using autologous chondrocytes in a biphasic three-dimensional collagen scaffold have been utilized in the USA or European countries, since 2003, although, they are not FDA-approved, yet (NCT0234869). Furthermore, Chondrosphere[®] (spherox) is another commercial scaffold-free product made of autologous chondrocytes which is an established treatment for large or full-thickness cartilage defects, but there is still a requirement to expand the clinical knowledge and long-term follow-up (Niemeyer et al. 2019).

Different results have been reported from stem cells used for cartilage regeneration. The nonacceptable results were believed to be happening because of the source of stem cells and the scaffold being used for cartilage tissue engineering. The complications with scaffolds included the lack support by the scaffolds of proliferation and differentiation of normal cells, poor cell survival, cell death, and low-cell penetration. Also, poor cell differentiation, improper cell distribution, and poor integration inside the host are common problems when it comes to cell transplantation methods (Ma et al. 2018; Ha et al. 2015; Lee et al. 2019).

Newly found TE cell-free approaches such as conditioned media and EVs tend to mimic microscopic and molecular *in vivo* environments and their goal is enhancing the natural processes of tissue repair and development. In this regard, the presence

and therapeutic function of EVs allow for clinical applications in cartilage repair and regeneration.

18.3.4 Extracellular Vesicles: A Promising Cell-Free Therapy for Cartilage Repair

EVs are membrane-bound vesicles which are secreted by all different types of cell types such as MSCs, monocytes, and embryonic stem cells that act as a mediator in cell-cell interaction through their lipid, protein, carbohydrate, and nucleic acid (RNA, DNA) cargo. In addition, EVs are also included in a few physiological processes such as development, cell differentiation, and angiogenesis, plus they are included in tissue repair (Liu et al. 2017). EVs exist in all body fluids, including milk, semen, urine, and blood (Hu et al. 2021; Erdbrugger et al. 2021). So far, the formation of hyaline cartilage reported in several preclinical studies following intra-articular injection of exosomes in an osteochondral defect animal model (Wang et al. 2017a; Wong et al. 2020; Zhang et al. 2022; Esmaeili et al. 2022; Sharun et al. 2022).

Some authors claimed that EVs have a more therapeutic effect than their parental cells when applied to articular damage animal models. For instance, Zavatti et al. showed amniotic fluid-derived EVs have more therapeutic effects than amniotic fluid stem cells (AFSCs) in cartilage defects (Zavatti et al. 2020). Likewise, Moon et al. indicated that the use of EVs derived from MSCs was the efficacious treatment strategy than MSCs in a rat stroke model study (Moon et al. 2019). However, other studies have shown that EVs have the same functional tissue repair ability as parental MSCs in animal models with skeletal defects (Qin et al. 2016). Different results of the therapeutic effect of EVs in comparison with parental cells shows at least they have the same effects as stem cells. These outcomes are related to the kind of diseases and approaches of EVs delivery. In conclusion, the distinct advantages of EVs cannot be ignored. For example, EVs are cell-free agents that have simpler storage needs, lack MHC I and MHC II antigens which complicate allogeneic transplantation, have low immunogenicity and no need for immunosuppressive drugs, are safe in clinical trials, and have no risk for malignant transformation or proliferation. Moreover, they are capable of crossing biological barriers like blood–brain barrier as well as the ability for specific targeting and bioengineering. In contrast, the most relevant risks associated with MSC therapy are promotion of the development of tumorigenic cells and cell homing at ectopic sites as well as genomic variability of stem cells at more proliferation (Bahr et al. 2020). The manipulation EVs with MicroRNA, Kartogenin, and hypoxia to improve therapeutic effects such as promotion of proliferation and avoiding apoptosis in chondrocytes or chondrogenic differentiation of MSCs has been reported (Rong et al. 2021; Xu et al. 2021).

Several clinical studies have applied EVs for different diseases such as COVID (Mazini et al. 2021; Gurunathan et al. 2021), renal fibrosis (Jin et al. 2021), and heart disease (NCT03984006). The first clinical study related to EVs for treatment of OA established in clinical [trial.gov](https://clinicaltrials.gov) is a phase I study to assess the safety of EVs-derived

allogeneic MSCs ($3\text{--}5 \times 10^{11}$ particles) produced in good manufacturing procedures (GMP)-facility. The EVs will be introduced through intra-articular injection in the knee of ten patients with mild to moderate symptomatic osteoarthritis and the follow-up will be up to 12 months (NCT05060107). However, the development of EVs approaches is still very novel and request more investigation. Many topics must be addressed such as standardization of EVs isolation, characterization and manipulation methods' hardness of large-scale production, limitation of high-yield products, short half-life in circulation as well as inadequate clinical evaluation studies, and drug loading efficiency.

18.3.5 In Vivo Characterization for Bioconstructs Before Initiation of Clinical Studies (Preclinical Studies)

Adaptation of quantitative results of implantation of biological constructs in preclinical animal models to the clinic requires advanced diagnostic tools. Mechanical, biochemical, and biological properties, plus neo-tissue regrowth and their remodeling, are evaluated to investigate the exact performance of the implanted bio-constructs. Here, we try to explain the most standard modalities of stern assessment of tissue-engineered constructs in defect models. Furthermore, general methods such as mechanical tests, histological scoring, and noninvasive imaging techniques are reviewed for the practical evaluation of grafted biomaterials in vivo.

18.3.6 Mechanical Testing of Cartilage Implants

Mechanical characterization of developing products to regenerate cartilage is critical for their translation and, eventually, clinical applications. Mechanical tests generally used to assess cartilage, have been typically destructive. Also, since it is difficult to maintain sterile conditions in mechanical tests due to physical contact with the specimen, they may not be suitable for in vivo cartilage evaluation, especially in cases where implantation is required (Marchiori et al. 2019; Patel et al. 2019; Mansour et al. 2016). In vivo, indentation testing is a useful compression test for the nondestructive way of determining the variation of compositional and mechanical properties of cartilage structures (Li and Herzog 2006; Lyyra et al. 1999; Lu et al. 2004; Julkunen et al. 2008). Mechanical properties such as degeneration, dynamic mechanical modulus, stiffness, and thickness of cartilage can be characterized by an indentation test along with arthroscopy (Brommer et al. 2006; Li and Herzog 2005; Svård et al. 2018) and imaging modalities such as magnetic resonance spectroscopy (MRS) (Namiranian et al. 2020; Nieminen et al. 2004), near-infrared spectroscopy (NIRS) (Prakash et al. 2019; Horbert et al. 2019), ultrasound (Viren et al. 2011; Kiviranta et al. 2008; Saarakkala et al. 2003), and X-ray (Lindburg et al. 2013; Coan et al. 2010). The results of indentation testing on extracted tissue in animal studies cannot be generalized to clinical studies; although ongoing efforts are underway to make this method less invasive to improve mechanical analysis and cartilage

regeneration. The size and shape of the indenter are important in the design of minimally invasive considerations (Lyyra et al. 1999; Chen et al. 2015; Mayr et al. 2013). For mechanical measurement, in addition to the indentation test, more standard techniques can be used to analyze the quality of the formed neocartilage. (Li and Herzog 2005; Kang et al. 2018). An optimistic noninvasive method that implements this approaches is electro-arthrography, which detects streaming potentials generated by cartilage during joint compression and early cartilage degeneration (Changoor et al. 2021; Novakofski et al. 2016). The formed cartilage's mechanical properties are location-dependent and significantly different between loaded and unloaded locations. However, indentation tests are not efficient in examining the mechanical properties of some areas of cartilaginous tissue such as radial cartilage or osteochondral interface (Antons et al. 2018). Generally, this work may serve as a direction for cartilage tissue engineers seeking to precisely survey the physical properties of their novel therapeutic strategies.

18.3.7 Evaluating Biomaterial–Host Interactions with Histological Scoring

Histomorphometry, used in conjunction with histopathological evaluation, is a well-suited and valuable tool for the semi-quantitative analysis of bone and cartilage histological specimens (Jackson et al. 2019). As there is no universal standard scoring system for bone analysis, the combination of several parameters based on the position and orientation of the section, the defect type, and the histological stains can be used for histological analysis. In 1987, the scoring methods for measuring bone models had been standardized. Those scoring systems has been updated in 2012 focusing on four main criteria including length, area, quantity, and distance (Revell 1983; Parfitt et al. 1987; Parfitt 1988; Dempster et al. 2013). To enhance the quality of regenerated tissue after biomaterial implantation, various scoring systems have been developed. O'Driscoll score is one of the common osteochondral scoring systems (Reinholz et al. 2004). There is also the Bern score (Rutgers et al. 2010), Outerbridge score (Wei and Dai 2021), ICRS, and Oswestry Arthroscopy Score (OAS) (Paatela et al. 2020). These scoring systems are commonly nonparametric and offer effects of bone and cartilage repair in vivo, in a semi-quantitative and subjective manner. The overall performance of those systems can be improved by proper statistical analysis (Rutgers et al. 2010), more accurate criteria to assess bone and cartilage repair (such as tissue growth rate, cell proliferation, and discount of vacant spaces) (Gerstenfeld et al. 2005) and increased quantification of histological samples with noninvasive imaging methods (consisting of MRI, microcomputed tomography (micro-CT), ultrasound, and polarized light microscopy) (Orth et al. 2015; Palmer et al. 2013). In cartilage histology, advanced MRI methods such as delayed gadolinium-enhanced MRI of cartilage (dGEMRIC), glycosaminoglycan (GAG)-specific chemical exchange saturation transfer (gagCEST), sodium MRI, and quantitative CTA, as well as T2 and T1rho mapping allow cartilage monitoring. They can track disease progression and formation of engineered tissue and detect

early cartilage changes before morphological changes occur (Oei et al. 2018; Huang et al. 2019; Bittersohl et al. 2015). Some of these factors provide the possibility of better comparison between studies to increase the efficiency of tissue engineering products.

18.3.8 Evaluating Biomaterial Interaction with Imaging

In vivo imaging techniques provide essential information on various aspects of engineered tissue structures postimplantation, as well as being able to provide insight into interactions between engineered tissues and the host. It has been estimated that those techniques have facilitated the design and evaluation of next-generation tissue engineering strategies. Generally, these techniques should be noninvasive and safe. Technically, they must have a high potential for tissue penetration and high spatial resolution. The ability to provide information on tissue-biomaterial interactions such as biocompatibility and degradation, besides integration with the host tissue, function, stability, and graft position are other features of in vivo imaging techniques (Shrestha et al. 2020; Teodori et al. 2017; Gil et al. 2019). Therefore, to obtain quantifiable diagnostic results of biomaterial-host interactions and the extent of tissue repair, carefully selected techniques and standardized methods must be used (Gil et al. 2019; Trachtenberg et al. 2015).

18.3.8.1 Magnetic Resonance Imaging (MRI)

Based on proton detection in tissues with broad application in bone and cartilage tissue engineering, MRI was introduced as the common noninvasive imaging technique (Xu et al. 2008; Chesnick et al. 2011; Takazawa et al. 2012). Within the application of a strong magnetic field to the sample that aligns the nuclei with the field, the MRI apparatus creates radio frequency pulses that change the magnetic field. As a result, the rearrangement of protons creates image contrast depending on the different materials, conditions, and different speeds. Indeed, according to pulse sequence, longitudinal (T1) or transverse (T2) magnetic relaxation time, diffusion weight, proton density, and magnetic transfer ratio (MTR), MRI can supply practical and anatomical data (Pancrazio et al. 2007; Plewes and Kucharczyk 2012). The lack of exposure to ionizing radiation and the possibility of repeated scans and consecutive studies with minimal harmful effects have made MRI an attractive method for assessing the growth rate and structural integrity of bone, cartilage, and subchondral bone tissue regeneration and repair assessment, as well as for the diagnosis of clinical pathologies (Hartwig et al. 2009; Bouxsein et al. 2010; Oei, and ROBINSON WH, GOLD GE. 2014).

Due to the recent advances in MRI techniques, new methods have been developed for accurate quantification and diagnosis of the biochemical composition of tissues. Recently developed MRI techniques including gadolinium-delayed cartilage MRI (dGEMRIC), sodium MRI, glycosaminoglycan (GAG)-specific chemokines, ultrashort echo time (UTE), and exchange saturation transfer (gagCEST), are used especially in engineering osteochondral tissue. In these methods, GAGs,

proteoglycans (PGs), and the content/direction of collagen are quantified and used as modern imaging biomarkers to assess morphological changes, degeneracy, and repair of cartilage and subchondral bone tissue. Despite the advantages of these methods, there has not been sufficient research on the relationship of these markers with tissue growth in engineered scaffolds and the evaluation of biomechanical and physiological performance, as well as the interactions between tissue repair and scaffold degradation (Huang et al. 2019; Bittersohl et al. 2015; Oei et al. 2014). The osteochondral junction and subchondral bone structure can also be investigated using UTE and Fat-suppressed 3D Spoiled Gradient Recall (3D-SPGR) techniques. With the detection of short T2/T2* relaxation times and high field strengths, these allow visualization of deeply calcified cartilage layers and hyaline cartilage respectively. However, MRI techniques for assessing bioconstructs in animal models, and particularly for translation to clinical studies, need to be standardized and require additional in-depth analysis (Oei, and ROBINSON WH, GOLD GE. 2014; Disler et al. 1995).

18.3.8.2 Microcomputed Tomography (Micro-CT)

Micro-CT is one of the nondestructive imaging methods that create three-dimensional cross-sectional images of the sample by collecting transmitted X-rays at different angles employing a multiarray detector. This approach has been widely used to visualize structures with a high concentration of minerals in hard tissues like bone, and also provides the possibility of studying the 3D structure of tissue engineering scaffolds and tracking the cells embedded in the scaffolds (Gil et al. 2019; Orhan 2020). In vivo monitoring of bone tissue engineering using micro-CT imaging has been widely used for various biomaterials and animal models, which allows imaging of the scaffold, old bone, and newly formed tissue segments (Potter et al. 2006; Zhang et al. 2010; Jones et al. 2007). Since the micro-CT technique is less sensitive for visualizing the contrast between different soft tissue structures because of the lack of mineral components, it is challenging to evaluate cartilage structures with this method. For this purpose, various contrast agents have been developed that are implanted in soft tissue scaffolds and have provided the possibility of imaging and measuring articular cartilage in animal models just like in human patients (Ji et al. 2011; Da et al. 2013; Xie et al. 2009; Aula et al. 2009). Detecting changes in the cartilage composition and quantitatively evaluating the structure of subchondral bone with contrast-enhanced computed tomography (CECT) is possible. Delayed CECT allows the detection of early degenerative changes by changing the diffusion of the contrast agent in the cartilage (Myller et al. 2017; Kokkonen et al. 2012; van Tiel et al. 2012). CECT, by using anionic, and more recently, cationic contrast materials, enables the imaging of cartilage by detecting the content of PGs and GAGs (Lusic and Grinstaff 2013; Bansal et al. 2011a, b). It has also recently been shown that the use of a mixture of two contrast agents and a CT technique with two X-ray energies for imaging human osteoarthritis cartilage has significant potential for the diagnosis of articular cartilage degeneration (Honkanen et al. 2020). The use of these techniques in the clinical monitoring of engineered bone and cartilage

tissue can maintain sensitivity to cartilage and bone morphology changes and also has an economic advantage compared to other imaging methods (Flynn et al. 2021).

18.3.8.3 Noninvasive Tracking and Monitoring

Although significant progress has been made in *in vivo* analysis methods, there are still many challenges in relating the biological activity of scaffolds and spatiotemporal modeling of delivery strategies of cells, nanoparticles, and growth factors for tissue formation. The primary goal of bone and cartilage tissue engineering is the functional restoration of damaged or degenerated tissue and new tissue formation. However, accurate assessment of tissue engineering constructs *in vivo* using noninvasive methods is crucial to comprehend how the delivered cells or biomolecules interact with damaged tissue and compare their performance with those *in vitro*. In the section that follows, the technologies used to noninvasively monitor growth factor retention and kinetics, scaffold degradation, and stem cell activity *in vivo* are briefly discussed (Trachtenberg et al. 2015).

18.3.8.4 Fluorescent Labeling

Fluorescent labeling, a popular and widely used technique for tracking cells and biomolecules, allows for the imaging of cell interactions, tissue functions, and the overall structure. The process of fluorescent labeling typically involves selectively binding a reactive derived from a fluorophore to a functional group that is present in the target biomolecule (Sahoo 2012). Additionally, fluorescence-based assays are beneficial experimental tools in tissue engineering research because they enable noninvasive and highly sensitive tracking of delivered cells and the ability to observe the processes of transplantation, survival, and tissue induction (van Gaalen et al. 2010; Leblond et al. 2010). In particular in bone tissue engineering, fluorescent labeling can be used to determine the initiation time and location of mineralization as well as the rate and direction of bone formation. This can be used to determine the ability to provide the appropriate scaffold for bone formation (van Gaalen et al. 2010). For example, it was demonstrated that labeling human placental mesenchymal stem cells (hPMSCs) with the red fluorescent dye PKH26 allowed for *in vivo* observation and evaluation of the mineralization and osteogenic potential of these cells using bone morphogenic proteins 2 (BMP-2)-tethered human lung fibroblast matrix (hFDM)-coated mesh scaffold after subcutaneous implantation in nude mice (Kim et al. 2015a). Also, to investigate cell migration inside tissues or 3D scaffolds, membranes can be labeled with vital dyes such as PKH dyes. It has been shown that labeling transplanted MSCs with the fluorescent dye PKH26 allows the tracking of proliferation, differentiation, and survival of these cells as well as their adhesion to the scaffold in full-thickness bone and subcutaneous implantation models in rabbit and minipig, as well as in the sheep joint cavity model (Tatebe et al. 2005; Chawla et al. 2007; Yang et al. 2008; Chen et al. 2005). Moreover, new fluorescence-based noninvasive systems have been developed for the possible monitoring of implant or scaffold degradation in tissue engineering. To track *in vivo* biological processes and the kinetics of scaffold degradation, scaffolds have been labeled in several studies with a variety of fluorescent probes, including proteins, artificial dyes, iron oxide

nanoparticles, and quantum dots (Dong et al. 2017; Nidhin et al. 2014; Yang et al. 2005; Mehwish et al. 2019; Vedhanayagam et al. 2021). In a mouse subcutaneous model, covalent binding of the fluorescent probe tetramethylrhodamine isothiocyanate (TRITC) to the chitosan scaffold backbone has shown a correlation between weight loss during scaffold degradation with a decrease in fluorescence intensity (Cunha-Reis et al. 2013). However, there are drawbacks to using fluorescence *in vivo*, including light bleaching, low resolution and exhibiting low tissue depth due to natural tissue autofluorescence, and unmeasurable signals due to the effect of cell number and efficiency on fluorescence intensity. For enhanced depth detection and physicochemical stability, systems using fluorescent nanoparticles and near-infrared fluorescence imaging have been developed. However, further research is needed because they have negative effects on cells (Jung et al. 2019; Lee et al. 2012; Kim et al. 2020). Therefore, it is crucial to create a standardized noninvasive fluorescent labeling technique to enhance the design of scaffold systems for a variety of clinical applications, as well as to better our knowledge of specific cellular processes underlying cell therapy.

18.3.8.5 Bioluminescent Imaging

In vivo bioluminescence imaging (BLI) is a noninvasive molecular imaging technique that works by detecting light emissions from biological samples. BL is a natural phenomenon in which photons of light are created biochemically through the catalytic oxidation of luciferin by luciferase enzymes (Kim et al. 2015b; Zambito et al. 2021). BLI techniques enable long-term cell tracking after implantation as well as the observation of cell survival, proliferation, and migration on different scaffolds due to their high detection sensitivity and increased signal-to-noise ratio. BLI can also be used to monitor a variety of processes involved in bone tissue engineering, including differentiation, apoptosis, inflammation, and vasculogenesis (De Boer et al. 2006; Degano et al. 2008). Firefly luciferase and renilla luciferase are two of the most widely used bioluminescent transgenic reporters that have been used to track cell proliferation, distribution, and migration in ectopic and orthotopic sites for bone and cartilage tissue engineering (Olivo et al. 2008; Takaku et al. 2014). However, BLI still has drawbacks such as not penetrating hard tissue and signal attenuation by surrounding tissues. Additionally, since BLI relies on living cells to emit light, imaging implants postharvest is also not possible (Degano et al. 2008; Allen et al. 2014). Enhancements to luciferase-luciferin systems have recently been made to enable more sensitive and in-depth imaging and monitor enzyme activity and other intracellular processes. This will increase the capacity of BLI for more challenging *in vivo* applications (Zambito et al. 2021).

18.3.8.6 Radiolabeling

Using some universal nuclear imaging approaches, e.g., scintillation and/or gamma counter, radiolabeling was developed to functionally monitor the kinetics, bioactivity, and concentration of growth factors. However, researchers have extended this approach for implanted cell tracking or scaffold biocompatibility in some studies. As an important advantage of radiolabeling, it can produce more stable signals for

in vivo analysis, and the data of this method can be quantified instead of fluorescence imaging or BLI (Gildehaus et al. 2011; Santo et al. 2013; Wang et al. 2010).

In the context of bone and cartilage tissue engineering, some of the radioactive dyes have been applied for radiolabeling of growth factors. In this regard, iodine-125 (¹²⁵I) is a commonly evaluated dye with a half-life of about 60 days. Within low levels of energy emissions, ¹²⁵I provides a harmless platform for conventional living assays through radiolabeling context (Ertl et al. 1970). Delgado, et al. (2006) validated a noninvasive method for in vivo evaluation of the release of growth factor from a local delivery system in bone (Delgado et al. 2006). By applying a probe-type gamma counter and a collimator, they managed to monitor the release kinetics of chitosan-loaded ¹²⁵I-labeled platelet-derived growth factors for 8 weeks in laboratory rat models of femur defect (Delgado et al. 2006). In another study, Kempen, et al. (2009) could efficiently monitor the in vivo retention of ¹²⁵I-labeled BMP-2 through a combination of single photon emission computed tomography (SPECT)/CT imaging and scintillation probes in bone tissue engineering (Kempen et al. 2009). The above-noted combination approach, combining the application of radioactivity measurements with radiographic and nuclear imaging, gave them a strong opportunity for simultaneous evaluation of both anatomical and functional characteristics. While radiolabeling has been introduced as one of the most powerful techniques for in vivo monitoring of growth factor delivery in tissue regeneration, the global application of this approach has been hindered due to some limitations, such as cost-benefit analyses. Furthermore, tracing the radioactive-labeled bioactive molecules from the implanted materials onto the targeted tissues would be challenging due to some issues, including the poor resolution of detectors (Vo et al. 2012).

18.3.8.7 Magnetic Particle Labeling

Magnetic particle imaging (MPI) is a modern, relatively fast imaging technique that allows high-resolution imaging at any depth and location by determining the concentration and distribution of magnetic tracer materials. The superparamagnetic iron oxide (SPIO) contrast agents frequently used in MRI cell tracking studies are also used in MPI (Panagiotopoulos et al. 2015; Saritas et al. 2013). SPIOs are the fundamental component of MPI, and their characteristics, such as their hydrodynamic diameter, the iron core of the particle anisotropy, particle composition, and coating, are useful in determining the MPI signal's strength, biocompatibility, and pharmacokinetics (Panagiotopoulos et al. 2015). Cell tracking with SPIO particles has been used in tissue engineering of both cartilage and bone because of their long detection times (up to 12 weeks) and minimal effects on cell viability (Heymer et al. 2008; Lalande et al. 2011). Since MPI directly images tracer particles while MRI does so indirectly, MPI is more sensitive to the detection of iron oxide. Additionally, because MPI measurements are performed in a magnetic field, the distribution of SPIOs can be seen in three dimensions with superb contrast and free of ionizing radiation. Therefore, it appears that MPI is appropriate for applications like in vivo targeted stem cell tracking, vascular imaging, and interventions (Borgert et al. 2012). However, some studies have revealed variations in the SPIO-labeled cells' ability to

differentiate as well as modifications in the morphology of the extracellular matrix (Farrell et al. 2008). Therefore, their use necessitates more thorough research on cell safety and function in tissue engineering studies.

18.4 Clinical Performance of Tissue-Engineered Constructs and Templates

18.4.1 Commercial or FDA-Approved Constructs

The autologous chondrocyte is the first cell-based product for cartilage treatment that received FDA approval in the USA (Brittberg et al. 1994). Furthermore, a later developed generation of ACI, matrix-supported MACI made by porcine collagen as a supportive matrix (Kon et al. 2012), was approved by the FDA in 2016 (Hambly 2011). Additional cell-based medicines have been clinically approved worldwide in the past few decades and are used for the regeneration of articular cartilage defects; however, the only cell and tissue engineering cartilage treatments that are currently approved are ACI and MACI in the USA (Table 18.1). There are already hundreds of more clinical trials in the works (OARSI White Paper 2018; Solheim et al. 2016). Retrieved on October 1, 2022; www.clinicaltrials.gov.

The commercial allogenic and autologous stem cell products which are approved processes are used for several disease treatments including graft versus host disease (GvHD), cartilage lesions, and low back pain (Wang et al. 2017b; Daly 2012; Dunavin et al. 2017; Murata and Teshima 2021). For instance, Chondrogen[®] which is made of hMSCs suspended in commercial sodium hyaluronan is used for treatment of cartilage injuries (Clinicaltrials.gov NCT00702741). The Stempeucel[®] is another allogenic bone marrow-derived MSC therapy that achieved first approval in India in 2019 for osteochondral treatment (Clinicaltrials.gov NCT01453738) (Gupta et al. 2016). ELIXCYTE[®] is the first allogenic adipose-derived hMSCs (ADSCs) with approval of the Taiwan FDA for intra-articular injection in patients who suffer from knee osteoarthritis (clinical trial study phase I/II) (Zheng et al. 2022; Chen et al. 2021). CARTISTEM[®] is an allogeneic umbilical cord blood-derived MSC regiment that is used for full-thickness cartilage defects in older patients (Lim et al. 2021).

Intra-articular transplantation or injection of MSCs is a slightly invasive technique in articular cartilage regeneration; however, the long-term fate and homing of cells must be considered (Nasiri et al. 2019). On the other hand, undifferentiated cells such as stem cells increase the risk of tumorigenicity and cell migration to the nonspecific site after cartilage repair (Huang et al. 2018). Therapeutic procedures for cartilage regeneration that have received clinical approval are dressed in Table 1 18.1.

Table 18.1 The list of therapeutic products for cartilage regeneration that have received clinical approval

Product name	Company	Cell type	Support material	Country of approval	Refs.
Carticel (ACI)	Vericel	Autologous chondrocytes	Implantation of periosteal flap	U.S. (FDA)	Vinardell et al. (2012)
Chondron™	Sewon Cellontech	Autologous chondrocytes	Fibrin gel	Korea (MFDS)	Nerem (2006)
Cartistem®	Medipost	Human umbilical cord allogeneic mesenchymal blood stem cells	N/A (injection into joint space)	Korea (MFDS)	Martincic et al. (2021)
ChondroCelect®	TiGenix	Autologous chondrocytes	Implantation of flap or commercially collagen membrane	E.U. (EMA)	Waktiani et al. (1994)
Novocart®	3D Aesculap Biologics	Autologous chondrocytes	Three-dimensional collagen-chondroitin sulfate scaffolds	Germany/Switzerland	Caplan et al. (1997)
JACC®	J-Tec	Autologous chondrocytes	Gel collagen	Japan (MHLW)	Vischer et al. (2021)
MACI®	Vericel	Autologous chondrocytes	Porcine type I/III collagen membrane	U.S. (FDA)	Shin et al. (2021)
Spherex (chondrosphere®)	co. don	Autologous matrix-associated chondrocytes	N/A (self-adhering)	E.U. (EMA)	Ha et al. (2021)
Ortho-ACI® (3rd gen. MACI)	Orthocell	Autologous chondrocytes	Porcine type I/III collagen scaffold	Australia	Liu et al. (2017)
Invossa™ (TissueGene-C)	Kolon Life Sciences	Allogeneic chondrocytes (Transduced retrovirally TGF-β)	N/A (injection into joint space)	Korea (MFDS)	Gille et al. (2010)

U.S. United States of America; FDA Federal Drug Administration; MFD8 Ministry of Food and Drug Safety; E.U. European Union; EMA European Medicines Agency; MHLW Ministry of Health Labor and Welfare. Products that are not prepared or not intended to be utilized with any biomaterials for supporting adhesion or localization are referred to as not applicable (N/A). ACI stands for articular chondrocyte implant. Chondrocyte treatment using matrix-supported autologous culture

18.4.2 Clinical Trials Founded on Validated Constructs

According to the findings of three clinical trials databases: [ClinicalTrials.gov](https://clinicaltrials.gov), the International Clinical Trials Registry Platform (ICTRP) of the World Health Organization (WHO), and EudraCT, very few cell and tissue engineering cartilage therapies have been clinically approved in the past few decades worldwide (Table 18.1). Numerous more therapies are participating in clinical trials right now (Makris et al. 2015; Negro et al. 2019). In this section, we go into greater depth on the clinical trials of each of the articular cartilage products listed in Table 18.1, which have been approved. Standard guidelines (Kraeutler et al. 2020; Mithoefer et al. 2011) and general assessments are used to determine the proportion of cartilage abnormalities that have been addressed. The main standard methods are briefly mentioned below.

18.4.2.1 VAS [Time Frame: x Days/Months]

The 100-mm Visual Analog Scale was used to gauge each subject's level of joint discomfort before and after the administration of the investigational medication. Scores on the 100-mm VAS range from 0 to 100. Worse joint discomfort is represented by higher values.

18.4.2.2 The score for Lysholm [Time Frame: x Days/Months]

A self-assessment tool called the Lysholm knee scoring scale measures the severity of typical knee symptoms like swelling, pain, strange sensations, and being able to squat or climb stairs. The score goes from 0 to 100, with 100 representing the absence of all symptom's disability. Excellent (95 to 100), good (84 to 94), fair (65 to 83), and bad (64) are the different categories for scores. The Lysholm score was used to gauge how well the knees were functioning in each subject before and after the administration of the IP. The postoperative change amount at the time frame from the baseline was shown as the outcome.

18.4.2.3 The Score According to KOOS [Time Frame: x Days/Months]

The Knee Injury and Osteoarthritis Outcome Score (KOOS) knee survey allows participants to rate their quality of life (QOL) and knee function. Each parameter's possible score has a range from 0 to 100, where 0 = extreme problems and 100 = no problems. Subjects responded to questions concerning knee-related symptoms, swelling, pain, impaired function, or changes in QOL with one of five possible answers, ranging from "never/not at all" to "always/extremely." Before and after the administration of the IP (investigational product), subjects performed the KOOS knee survey. The postoperative change amount from the baseline after the period was provided as the outcome.

18.4.2.4 IKDC Score [Duration: x Days/Months]

Before and after the IP was administered, subjects evaluated how their knees were functioning using the IKDC subjective knee evaluation. The evaluation is made to see whether symptoms, function, and athletic activity related to knee disability have

improved or worsened. The score goes from 0 to 100, with 100 representing complete freedom from restrictions on daily or athletic activity and the lack of symptoms. Before IP administration and 24 months post IP administration, an exploratory IKDC assessment was conducted. The postoperative change amount from the baseline at Month 24 was presented with the results.

It should be noted that the eligibility requirements are the same for all prospective clinical trials and consist of the following: participants must have at least 18 years of age, be of both sexes, and be in good health conditions. Additionally, the following criteria were shared by all clinical trials that have been done in the area of articular cartilage defects (Engen et al. 2010).

18.4.3 Inclusion Criteria

Patients having a single focal, full-thickness knee cartilage lesion that is intended for treatment (ICRS Grade 3 or 4) due to aging, trauma, or degenerative disorder could enter the clinical trials. The complete inclusion and exclusion criteria for patients entering into cartilage repair clinical trials with approved cellular products are listed in Table 18.2.

18.4.3.1 Carticel®

In this study, patients with knee articular cartilage abnormalities who previously underwent non-Carticel surgical therapy are evaluated prospectively, longitudinally, across many centers, and within hospitals. Patients who satisfied the requirements for enrollment were added to the trial. Patients receive follow-up care after Carticel implantation every 6 months for up to 48 months. About 12 million autologous cultured chondrocytes are transplanted into the defect in each Carticel vial, and the periosteal flap secures the implant. The Kerlan Jobe Orthopedic Clinic in Los Angeles, California, the Naval Medical Center San Diego in San Diego, California, the Santa Monica Orthopedic Group in Santa Monica, California, the United States, and 25 other centers that are fully listed at ClinicalTrials.gov participated in this clinical trial, which was sponsored by the Vericel Company. The evaluations carried out include Evaluation of mean change in the KOOS and Modified Cincinnati Score Change [Time Frame: Baseline, 6-month, 12-month, 24-month, 36-month, 48-month] from baseline. Change from Baseline in the KOOS, which has five sub-categories: pain, activities of daily living (ADL), QOL, symptoms, and sport [Time Frame: 12, 24, 36, 48 months]. In general, the obtained results of the treatment by Carticel were evaluated as acceptable (Guo et al. 2018).

18.4.3.2 Chondron™

Patients with knee cartilage abnormalities underwent a clinical trial to assess the long-term efficacy and Chondron safety (Autologous cultured chondrocyte) for 48 weeks and an additional 96 weeks. There are 24 participants in this open trial. After screening the subjects who grant their consent, those who fit the trial's requirements will receive Chondron via transplant. Subjects are required to adhere

Table 18.2 The complete list of inclusion and exclusion criteria for patients entering into cartilage repair clinical trials with approved cellular products

Inclusion criteria	Exclusion criteria
18 years of age	Asymptomatic patients who had previously received treatment
The articular cartilage lesion is more than 2 cm ² in size	Osteonecrosis and avascular necrosis
Swelling, tenderness, and active range of motion $\leq$ Grade II	Inflammatory or autoimmune joint disease
Joint pain: 20–60 mm on VAS (visual analog scale) at the time of screening	Recent history of illness lasting 6 weeks
Appropriate blood coagulation, kidney and liver function laboratory parameters: PT (INR) < 1.5 , APTT $< 1.5 \times$ control Creatinine ≤ 2.0 mg/dL Albumin $\leq$ trace in urine dipstick test Bilirubin ≤ 2.0 mg/dL, AST/ALT ≤ 100 IU/L	Within the last 6 weeks, either surgery or radiation treatment
Ligament instability $\leq$ Grade II	Serious medical conditions that, if present, would make surgery inadvisable, as evaluated by the researcher
Alignment of the lower extremities to the neutral weight-bearing axis within 5°	Presently expecting or breastfeeding
Over 5 mm of the meniscal rim is still present and you haven't had meniscal surgery in the last 3 months	Epilepsy, psychotic conditions, or a history of such conditions
Having the capacity and desire to fully engage in the postoperative rehabilitation program	Currently abusing alcohol (more than 10 drinks per week) and/or regularly exposing oneself to other addictive substances; currently a smoker
Before beginning any of the screening processes, the subject is made aware of the exploratory nature of the study, voluntarily consents to participate in it, and signs an informed consent form that has been authorized by the IRB	Rheumatoid arthritis and other chronic inflammatory arthritis
Less than 35 kg/m ² for body mass index (BMI)	Any more clinical trials during the last 4 weeks
	Administered immunosuppressants within the last 6 weeks, such as azathioprine or cyclosporin A
	Ligament instability $>$ Grade II
	Uncorrected significant lower extremity malalignment (i.e., $> 5^\circ$)
	(sub-) Total meniscectomy (< 5 mm rim remaining)
	Injections of viscosupplementation or corticosteroids into the afflicted knee over the previous 3 months

to the lead investigator's directions during the trial time. Subjects will make 8* hospital visits regularly inclusive of hospitalization. During these visits, doctors will examine subjects, blood tests, x-rays, MRIs, observation of the unaided eye, and arthroscopy will be carried out.

*Sewon Cellontech Company supported this clinical investigation, which was carried out at Inha University Hospital in Incheon, Republic of Korea, and Ewha Womans University Mokdong Hospital in Seoul, Republic of Korea.

The evaluations carried out include: change in discomfort measured using a 100 mm VAS [48 weeks following surgery], IKDC modification, change in KSS, ICRS, KOOS, MRI, and mMOCART results [Time Frame: Baseline, 48 weeks following surgery, and 12-, 24-, and 96-weeks following surgery]. The primary comparison will be between the afflicted knee's grades at baseline and 48 weeks following surgery (Efe et al. 2012). In general, the obtained results of the treatment by Chondron were evaluated as acceptable.

18.4.3.3 Cartistem®

This study aims to assess the efficacy and safety of Cartistem®, which is a cell therapy product, produced for treating articular cartilage abnormalities of the knee caused by aging, trauma, or degenerative illnesses. The single-dose cellular therapy agent Cartistem®, which combines sodium hyaluronate and human umbilical cord blood-derived MSCs, is designed to be used in regenerating cartilage in human subjects with knee cartilage abnormalities. The cartilage Restoration Center, RUSH University Medical Center, Chicago, Illinois, the Cartilage Repair Center, and Brigham and Women's Hospital, Chestnut Hill, Massachusetts, undertook this research experiment under the sponsorship of the Medipost firm.

The evaluations that they carried out include investigations of change in discomfort measured using VAS, Lysholm, KOOS, and IKDC scores before and during 12 and 24 months after IP (investigational product) administration (Zhang et al. 2020). In general, the obtained results of the treatment by Cartistem® were evaluated as acceptable.

18.4.4 MACI®

To compare the effectiveness of MACI (matrix-applied characterized autologous cultured chondrocytes) implants with arthroscopic microfracture when it comes to treating the articular cartilage defects in the patients' knees from 18 to 55, this prospective, randomized, open-label, multicenter study was created. Before being randomly assigned to the research treatment, every qualified patient deemed acceptable for inclusion will have a cartilage biopsy obtained. Then, during the index arthroscopy operation, to receive either a MACI implant or microfracture therapy, eligible patients will be assigned randomly.

Within 4–8 weeks into index arthroscopy, patients who were randomly assigned to receive treatment with a MACI implant will return to have the chondrocyte implantation operation done by an arthrotomy. During the index arthroscopy,

patients who were randomly assigned to microfracture would have the surgery done. Over the course of a 2-year follow-up, patients will have periodic postarthroscopy and postarthrotomy evaluations. Evaluations include arthroscopic and histologic examination, magnetic resonance imaging, functional and pain results, and adverse events (MRI).

The evaluations carried out include investigations of change in discomfort measured using KOOS, IKDC Score, mean microscopic overall assessment score, and MRI evaluation of defect fill before and at week 104 after receiving MACI. For participants who had an MRI with a defect fill rate greater than 50%, unbiased central reviewer that was unaware of the participant's therapy evaluated the MRI data. The results of this completed study were published (Saris et al. 2014). Table 18.3 summarizes the clinical trial conditions on commercialized products.

18.5 Concluding Remarks and Future Trends

Engineered cartilage is a promising technique for complete articular cartilage repair, osteochondral defects, and restoration of joint function. Despite numerous *in vitro* and *in vivo* studies on MSC-based cartilage repair, there are still many unsolved challenges due to the lack of properly translating results from *in vitro* to clinical trial studies. Instead of optimizing the current methods that have been investigated in laboratory conditions, scientists tend to use new scaffolds and cells, which has resulted in the lack of, a standard and optimal cell and scaffold material for articular cartilage repair after several decades. Due to the restriction on chondrocyte usage, many scientists believed MSCs are ideal cells for tissue-engineered cartilage. Also, there are still possible concerns about the use of MSCs, such as tumorigenicity of MSC transplantation, immune rejection of allogeneic MSCs, and disease transmission. Unfortunately, in the long-term follow-up of large-scale clinical trials, the results of cartilage tissue regeneration and the function of MSC-treated patients are still controversial. For instance, some systematic studies and meta-analyses have reported significant improvement in MSC engraftment into cartilage defects based on multiple assessments; however, other studies have reported no significant improvement based on other scores or analyses. In many cases, the different results of cartilage repair and improved function of patients compared to the control group are reported which have not been carefully selected. In addition, the effect of cell sources, cell doses, and combination treatments with auxiliary materials such as hyaluronic acid (HA), collagen, and PRP cannot be ignored. Furthermore, by introducing a standard criterion for choosing the type of treatment strategy that is appropriate for the specific conditions of the patient, they can own the maximum benefits of their treatment methods.

Cell-free approaches such as exosome therapy are believed not to transfer the possible risk of MSCs such as immunogenicity and tumorigenic risks, and therefore, are great alternatives for MSCs in cartilage-tissue engineering although this strategy is indirectly dependent on cells. It has been proven that exosomes that are secreted by MSCs from various tissue sources are capable of repairing articular cartilage defects

Table 18.3 Summary of clinical trial conditions on commercialized products

Product name	Condition/disease	Intervention/treatment	Patients	Intervention model	Official title	Study date
Carticel®	Articular Cartilage Defect	Biological: Carticel (autologous cultured chondrocyte) implantation	126	Single group assignment	A prospective, longitudinal within-patient evaluation of the effectiveness (durability) of Carticel® (autologous cultured chondrocytes) compared to non-Carticel surgical treatment for articular cartilage defects of the knee	2000–2005
Chondron ^{MT}	Osteoarthritis Traumatic Arthritis	Device: Chondron Implantation	24	Single group assignment	This postmarketing surveillance to evaluate the efficacy and safety of a CHONDRON (autologous cultured chondrocyte) in patients with cartilage defects in their knees	2012–2016
Cartistem®	Degeneration articular cartilage knee	Biological: allogeneic umbilical cord blood-derived mesenchymal stem cells	12	Single group assignment	Evaluation of safety and exploratory efficacy of CARTISTEM®, a cell therapy product for articular cartilage defects: a phase I/IIa clinical trial in patients with focal, full-thickness grade 3–4 articular cartilage defects of the knee	2013–2017
MACI®	Articular cartilage defect	- Biological: autologous cultured chondrocytes on porcine collagen membrane - Procedure: microfracture	144	Parallel assignment	A prospective, randomized, open-label, parallel-group, multicenter study to demonstrate the superiority of MACI® versus arthroscopic microfracture for the treatment of symptomatic articular cartilage defects of the Femoral C	2008–2012

in animals, but more clinical applications need to be reported. There is also another type of cell-free strategy that utilizes some scaffolds which are completely independent of cells, although their outcome needs more evaluation.

Recent obstacles and their possible solutions are:

1. Introduction of a safe, effective, noninvasive, and ethically permitted cell source: such as bone marrow mesenchymal stem cells.
2. Finding appropriate scaffold materials based on natural articular cartilage matrix material for cell-based or cell-free tissue engineering methods.
3. Finding the best cell-free compounds or methods that do not require cell processing.
4. Finally, standardizing the method of investigating the clinical trial outcomes, considering the limitations that some of these methods may have in clinical studies.

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